UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS
DEPARTAMENTO DE FARMACOLOGIA
CAMPUS DE BOTUCATU

VIAS DE SINALIZAÇÃO ENDOTELIAL α_2, AT_1 E AT_2 SOBRE A REATIVIDADE DA AORTA DE RATOS APÓS CONSUMO DE ETANOL E EXPOSIÇÃO AO ESTRESSE, ISOLADAMENTE E EM ASSOCIAÇÃO

RAFAELA DE FÁTIMA FERREIRA BAPTISTA

Tese apresentada ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu para obtenção do título de Doutor em Ciências Biológicas (Área de Concentração: Farmacologia)

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Sumário

Resumo

Introdução: o estresse e o consumo de etanol são importantes fatores de risco cardiovascular. **Objetivos:** avaliar os efeitos da exposição ao estresse e do consumo de etanol, isoladamente e em associação sobre a pressão arterial e a reatividade da aorta torácica *in vitro.* **Métodos:** ratos Wistar machos adultos (8-10 por grupo) foram separados em grupos: controle, etanol (20% de etanol na água de beber por 6 semanas), estresse (contenção 1h/dia 5 dias/semana durante 6 semanas) e etanol/estresse (ambos procedimentos em associação). A pressão arterial sistólica caudal (PAS), a concentração plasmática de corticosterona e a capacidade antioxidante do plasma foram avaliadas. Curvas concentração-efeito à noradrenalina na ausência ou presença de ioimbina (bloqueador-α_2), L-NAME (inibidor da sintase do óxido nítrico), ou indometacina (inibidor da ciclooxigenase); à fenilefrina; e à angiotensina II na ausência e presença de losartan (bloqueador-AT_1), PD123-319 (bloqueador-AT_2), L-NAME, ou indometacina foram obtidas em anéis de aortas intactas e desnudadas de endotélio. Concentração efetiva 50% (CE50) e resposta máxima (RM) foram comparadas entre os grupos utilizando MANOVA/Tukey. $P < 0,05$ foi considerado estatisticamente significativo. **Resultados:** a exposição ao estresse, isoladamente e em associação ao consumo de etanol aumentou a PAS. Estresse ou estresse em associação aumentou as RM à noradrenalina em aortas intactas. Esta hiperreatividade foi abolida pela remoção do endotélio e pela presença de indometacina ou ioimbina, mas não foi alterada pela presença de L-NAME. Por sua vez, o consumo de etanol isoladamente não alterou a reatividade à noradrenalina. As respostas à fenilefrina em aortas com e sem endotélio e à noradrenalina em aortas sem endotélio permaneceram inalteradas independentemente do protocolo. Etanol e estresse, isoladamente e em associação, não alteraram a reatividade da aorta intacta à angiotensina II. PD123-319 diminuiu a RM à angiotensina II na aorta intacta de ratos dos grupos etanol e etanol/estresse comparado ao grupo controle na presença de PD123-319. Losartan aumentou a RM à angiotensina II na aorta intacta de ratos dos grupos estresse e etanol/estresse comparado ao grupo controle na presença de losartan. Nenhum dos protocolos alterou a reatividade da aorta desnuda à angiotensina II, independentemente da presença de losartan ou PD123-319. A presença de indometacina ou L-NAME não alterou a reatividade à angiotensina II de aortas intactas dos diferentes grupos experimentais. Tanto

a capacidade antioxidante do plasma quanto a concentração plasmática de nitrito/nitrato total não foram diferentes entre os grupos experimentais. A concentração plasmática de corticosterona mostrou-se elevada nos grupos estresse e etanol/estresse. **Conclusões:** o aumento de RM à noradrenalina induzido pela exposição ao estresse, isoladamente e em associação, mostrou-se dependente da integridade do endotélio vascular e envolve a liberação de prostanóides vasoconstritores via estimulação de adrenoceptores alfa-2 endoteliais. Ainda, o consumo de etanol, isoladamente e em associação alterou a sinalização endotelial via receptores-AT_1, sem alterar PAS. Por sua vez, o estresse, isoladamente e em associação alterou a sinalização endotelial via receptores-AT_2, e, paralelamente, aumentou a PAS. O conhecimento de tais alterações vasculares induzidas pelo estresse e/ou etanol pode contribuir para a compreensão dos efeitos cardiovasculares adversos da exposição ao estresse e do consumo de etanol em seres humanos.

Palavras-chave: consumo de etanol; estresse; reatividade vascular; endotélio, receptores α_2, AT_1 e AT_2

Abstract

Introduction: stress and ethanol consumption are important cardiovascular risk factors. **Objectives:** to evaluate the effects of stress exposure and ethanol consumption, alone and in association on blood pressure and thoracic aorta reactivity *in vitro*. **Methods:** Wistar adult male rats (8-10 per group) were separated into groups: control, ethanol (20% ethanol in their drinking water for 6 weeks), stress (restraint 1h/day 5 days/week for 6 weeks) ethanol/stress (both procedures in association). Caudal systolic blood pressure (SBP), plasma corticosterone and plasma antioxidant capacity were evaluated. Concentration-effect curves to noradrenaline in the absence or presence of yohimbine (α_2-blocker), L-NAME (inhibitor of nitric oxide synthase) or indomethacin (cyclooxygenase inhibitor); phenylephrine; and angiotensin II in the absence and presence of losartan (AT_1-blocker), PD123-319 (AT_2-blocker), L-NAME or indomethacin were obtained from the intact and denuded-endothelium aortas. 50% effective concentration (EC50) and maximum response (MR) were compared between groups using MANOVA/Tukey. $P < 0.05$ was considered statistically significant. **Results:** exposure to stress, alone and in association with ethanol consumption increased SBP. Stress and stress in association increased the MR to noradrenaline in intact aortas. This hyperreactivity was abolished by both removal of the endothelium and the presence of indomethacin or yohimbine, but was not changed by the presence of L-NAME. In turn, the consumption of ethanol alone did not alter the reactivity to noradrenaline. Responses to phenylephrine in aortas with and without endothelium and to noradrenaline in aortas without endothelium remained unchanged regardless of protocol. Ethanol and stress, alone and in association, did not alter the reactivity of intact aorta to angiotensin II. PD123-319 decreased

MR to angiotensin II in intact rat aortas of ethanol and ethanol/stress groups compared to the control group in the presence of PD123-319. Losartan increased the MR to angiotensin II in intact rat aortas of stress and ethanol/stress groups compared to the control group in the presence of losartan. None of the protocols modify the reactivity of the aortic rings to angiotensin II, regardless of the presence of losartan or PD123-319. The presence of indomethacin and L-NAME did not change the reactivity to angiotensin II of intact rat aortas from the different experimental groups. Both the antioxidant capacity of plasma as well as the plasma concentration of nitrite/nitrate did not differ between the experimental groups. The plasma corticosterone was high in stress and ethanol/stress groups. **Conclusions:** The increased MR to noradrenaline observed in aortas from rats exposed to stress, alone and in association, was shown to be dependent on the integrity of the endothelium and involves the release of vasoconstrictor prostanoid via stimulation of endothelial alpha-2 adrenoceptors. Still, ethanol consumption, alone or in association, altered endothelial signaling via AT_1-receptors without changing SBP. In turn, stress, alone and in association, altered endothelial signaling via AT_2-receptors, and at the same time increased the SBP. The knowledge of such vascular changes induced by stress and/or ethanol may contribute to the understanding of adverse cardiovascular effects of exposure to stress and ethanol consumption in humans

Keywords: ethanol consumption; stress; vascular reactivity; endothelium, α_2-, AT_1- and AT_2-receptors

Introdução

O alcoolismo é considerado um problema de sáude pública em escala mundial. Usado socialmente ou abusivamente, o álcool é a droga mais consumida no mundo. O impacto do consumo de etanol sobre a saúde da população mundial tem sido tema de preocupação e investigações constantes da Organização Mundial da Saúde (WHO, 2014). Segundo dados recentes, aproximadamente 3,3 bilhões de indivíduos morrem anualmente devido ao consumo de bebidas alcoólicas no mundo, ou seja cerca de 5,9% de todas as mortes em 2012 (WHO, 2014). Ainda, 5,1% das doenças globais é atribuído ao consumo de alcool. No Brasil 12,3% da população entre 12 e 65 anos de idade preenchem critérios para a dependência ao etanol e cerca de 75% já beberam pelo menos uma vez na vida (SENAD, 2007).

O consumo de etanol pode causar alterações cardiocirculatórias que contribuem para o desenvolvimento de doenças cardiovasculares (TIRAPELLI et al., 2007; 2008). Entretanto, a severidade do dano cardiovascular causado pelo consumo de etanol depende de sua intensidade e duração. A literatura relata que baixas concentrações de etanol são benéficas para a função das células endoteliais e podem, deste modo, diminuir a pressão arterial (TODA; AYAJIKI, 2010). Atualmente são reconhecidos diversos fatores que levam à diminuição da pressão arterial após baixas doses de etanol como, por exemplo, a produção aumentada de óxido nítrico (TODA; AYAJIKI, 2010), aumento na expressão da enzima óxido nítrico sintase endotelial, aumento na resposta vascular à acetilcolina (vasodilatador dependente do endotélio) (KLEINHENZ et al., 2008*),* bem como aumento dos níveis da enzima óxido nítrico sintase induzível (TIRAPELLI et al., 2008).

Por outro lado, altas doses de etanol podem determinar um prejuízo na função e viabilidade das células endoteliais (TODA; AYAJIKI, 2010). Muitos estudos mostram que o consumo crônico de etanol acarreta alterações significativas das funções cardíacas e circulatórias, figurando como um importante fator de risco no desenvolvimento de doenças cardiovasculares, como por exemplo, a hipertensão arterial (SUN; MAYHAN 2001; HUSAIN et al., 2005, 2008; TIRAPELLI et al., 2007, 2008a). De fato, observou-se aumento da pressão arterial em homens e mulheres que consomem etanol regularmente, independentemente de outros fatores associados como o fumo e a obesidade (MILLER et al., 2005). Outros estudos também mostraram que o consumo regular de etanol, isoladamente, eleva a pressão arterial de humanos (ABDEL-RAHMAN; MERRILL; WOOLES, 1987; MOORE et al., 1990; PUDDEY; BEILIN, 2006). Recentemente, demonstrou-se que ratos Fisher tratados com solução de etanol a 20% por 12 semanas apresentam elevação da pressão arterial (HUSAIN et al., 2010). Neste sentido, Tirapelli et al. (2007) também observaram aumento da pressão arterial sistólica e diastólica de ratos Wistar após a segunda e sexta semanas de tratamento com etanol a 20%. A elevação da pressão arterial associada ao consumo crônico de etanol pode ser devida à diminuição do relaxamento induzido por agentes vasodilatadores e/ou a um aumento de reatividade para agentes vasoconstritores (CRIQUI, 1987). Respostas diminuídas à acetilcolina foram relatadas em aorta (HUSAIN et al. 2010) e em leito mesentérico (TIRAPELLI et al., 2008) de ratos tratados com etanol a 20%. Contrariamente, alterações de resposta vasodilatadora à acetilcolina não foram observadas em aorta de ratos tratados com etanol na concentração de 10% e 20% (LAPIDO et al., 2002; TIRAPELLI et al., 2007) e

em carótida e artéria mesentérica de ratos tratados com etanol a 20% (TIRAPELLI et al., 2007).

O etanol parece não influenciar apenas as ações de agentes vasolilatadores. Tirapelli et al. (2006) observaram um aumento da resposta contrátil induzida pela fenilefrina em aorta de ratos tratados com etanol 20% por 2, 6 e 10 semanas. Aumento de reatividade à noradrenalina também foi observado em aortas de ratos Sprague-Dawley submetidos ao tratamento crônico com etanol 10% por 5 meses (LADIPO et al., 2002). Além disso, aumento das respostas contráteis à noradrenalina e à fenilefrina foi observado em artéria mesentérica de ratos tratados com etanol a 36% por 18 semanas (HATTON et al., 1992) e em leito mesentérico de ratos tratados com etanol a 20% por 6 semanas (TIRAPELLI et al., 2008a).

A literatura, no entanto, não é uníssona no que diz respeito aos efeitos do etanol sobre as ações de agentes vasoconstritores. Neste sentido, tem sido sugerido que as respostas contráteis de aortas de ratos à fenilefrina não são modificadas pelo consumo de etanol por 12 semanas (UTKAN et al., 2001; HUSAIN et al., 2008). Por outro lado, o consumo de etanol por 10 semanas levou a uma diminuição da potência da aorta de ratos à fenilefrina (STRICKLAND; WOOLES, 1988). Estas informações conflitantes reforçam a necessidade de se compreender melhor os efeitos do consumo crônico de etanol sobre a resposta vascular.

Alguns estudos descrevem, ainda, que o consumo crônico de etanol induz ativação do sistema renina-angiotensina-aldosterona (WAKABAYASHI; HATAKE, 2001). O sistema renina-angiotensina-aldosterona é um sistema endócrino que tem como um de seus principais efetores a angiotensina II, cujos

efeitos se dão pela interação com receptores específicos, subtipo 1 (AT_1) e subtipo 2 (AT_2) (LEUNG, 2004).

A angiotensina II provoca constrição da parede vascular por meio de uma ação direta nas células do músculo liso, mas este efeito pode ser modulado pela ação da angiotensina II em células endoteliais. Semelhantemente as células musculares lisas, as células endoteliais apresentam receptores AT_1 e AT_2, que estão envolvidos na liberação de fatores de relaxamento e/ou contração derivados do endotélio (PUEYO; MICHEL, 1997). A predominância relativa de uma ou outra via de receptores-AT determina a ação final da angiotensina II nestas células.

Em vários leitos vasculares, a ativação de receptores AT_1 e AT_2 leva a efeitos antagônicos, promovendo um contrabalanço nas respostas vasoativas (FYHRQUIST; SAIJONMAA, 2008). Isto fica evidente na aorta de ratos que tiveram o gene para o receptor AT_2 suprimido, na qual a resposta contrátil à angiotensina II foi maior em comparação a do animal controle (AKISHITA et al., 1999). Ainda, o bloqueio dos receptores-AT_1 diminui, enquanto o bloqueio AT_2 aumenta a pressão arterial em ratos, indicando novamente um contrabalanço na resposta à angiotensina II (MUNZENMAIER; GREENE, 1996). Assim, a sinalização AT_2 é antagônica à sinalização AT_1, e resulta na formação de bradicinina e óxido nítrico (RUSH; AULTMAN, 2008). Por sua vez, a sinalização AT_1 resulta em aumento na produção de superóxido e aumento na geração de fatores de contração derivados do endotélio, como a endotelina-1 (TODA; AYAJIKI, 2010; MACKENZIE, 2011).

A ação da angiotensina II inclui também a amplificação das ações do sistema nervoso autônomo simpático (MALIK; NASJLETTI, 1976; KAWASAKI;

CLINE; SU, 1984). Sendo assim, a angiotensina II é essencial para a regulação do tônus vascular e da pressão arterial (RYAN et al., 2004). Além disso, tem um papel fundamental no controle e integridade funcional dos vasos sanguíneos e pode ter importante papel no processo fisiológico e fisiopatológico das doenças cardiovasculares (TOUYZ; SCHIFFRIN, 2000). Paralelamente à clássica formação de angiotensina II descrita no meio circulatório, tem sido demonstrada a formação de angiotensina II nos tecidos vasculares, onde provavelmente exerce ações parácrinas e/ou autócrinas promovendo, entre outros efeitos, a modulação das ações dos agonistas simpatomiméticos. Vale ressaltar, neste sentido, que o aumento da pressão arterial de ratos tratados cronicamente com etanol foi relacionado ao aumento dos níveis de angiotensina II no plasma e na aorta desses animais (HUSAIN et al., 2008).

A ativação dos receptores AT_1 pela angiotensina II, paralelamente à ação vasoconstritora direta, leva à ativação da fosfato dinucleotídeo adenina nicotinamida oxidase (NADPH). A NADPH é a principal mediadora para a formação de espécies reativas de oxigênio (EROs) na parede vascular (SIRKER et al., 2007). Atualmente é consenso que o aumento das EROs é um importante mecanismo da disfunção vascular. Nesse contexto, Sun e Mayhan (2001) observaram que o etanol induz estresse oxidativo e reduz o relaxamento de artérias cerebrais mediado pelo óxido nírico, sendo esse efeito revertido por antioxidantes. Ainda, a biotransformação do etanol no fígado pode gerar EROs que aumentam sua concentração quando o consumo de etanol é crônico (DAS; MUKHERJEE; VASUDEVAN, 2012).

O comprometimento da função endotelial, entretanto, não envolve necessariamente geração de EROs. Estudos clínicos e experimentais têm mostrado que o consumo crônico de etanol interfere com a produção e/ou liberação de óxido nítrico das células endotelias (SLOMIANY B.; PIOTROWSKI; SLOMIANY A., 1998; PUDDEY et al. 2001; HUSAIN et al., 2005). No endotélio vascular o óxido nítrico é sintetizado a partir da enzima óxido nítrico sintase endotelial dependente de Ca^{+2} e calmodulina (MICHEL; FERON, 1997). A produção de óxido nítrico pelo endotélio é criticamente dependente da função da óxido nítrico sintase endotelial e o consumo crônico de etanol em ratos promove redução da sua expressão em aorta, diminuindo a biodisponibilidade de óxido nítrico (HUSAIN et al., 2008). Corroborando estes achados, Toda e Ayajiki (2010) também relataram que a ingestão crônica de etanol induz disfunção endotelial associada à redução na biodisponibilidade de óxido nítrico.

Sabe-se, de longa data, que condições estressogênicas contribuem para o alcoolismo (POHORECKY, 1981). Etilistas diferem nas razões para o consumo de álcool, mas frequentemente ingerem álcool para diminuir a ansiedade e/ou estresse (POHORECKY, 1981). Outros estudos mostram que o nível basal de ansiedade tem papel importante na vulnerabilidade para ingestão de etanol (SPANAGEL et al., 1995).

A literatura relata que a exposição a agentes estressores pode deflagrar processos patológicos em muitos sistemas distintos do organismo, e que o sistema cardiovascular é particularmente vulnerável (CAPAZ; DE MORAES, 1988; CORDELLINI; VASSILIEFF, 1998; NAVARRO-OLIVEIRA; VASSILIEFF; CORDELLINI, 2000; ROZANSKY, et al. 2006). As ações do estresse crônico no

sistema cardiovascular implicam muitas vezes em doenças cardiovasculares como aterosclerose, hiperplasia na camada medial íntima da parede vascular (BLACK; GARBUTT, 2002; BRYDON; MAGID; STEPTOE, 2006), elevação de lipídeos no plasma, elevação dos níveis de fibrinogênio e elevação da pressão arterial (GE et al., 2007; ESLER, 2008). Assim, a literatura aponta o estresse crônico como fator de risco significante para morbidade e mortalidade cardiovascular em adultos (EVERSON-ROSE; LEWIS, 2005). Entretanto, a severidade dos danos cardiovasculares determinados pela exposição ao estresse depende da natureza do agente estressor, bem como, da sua intensidade e duração (HJEMDAHL, 2002).

Nas últimas décadas, o esclarecimento das vias neurais e metabólicas envolvidas no estresse vem colaborando para o entendimento de processos fisiopatológicos. As respostas mais comuns ao estresse envolvem a ativação do sistema nervoso simpático, do eixo hipotálamo-hipófise-adrenal (MCEWEN, 1998) e também do sistema renina-angiotensina-aldosterona (VIAU; MEANEY, 1991; HJEMDAHL, 2002). Como consequência disto, pode-se constatar elevação dos níveis sanguíneos tanto de corticosterona quanto de catecolaminas e angiotensina II (YANG et al., 1993; KVETNANSKY; LU; ZIEGLER, 2013). Devem-se considerar ainda, relatos da literatura mostrando que a ativação de receptores AT_1 pela angiotensina II durante o estresse atua de forma a intensificar o estresse oxidativo via ativação de NADPH oxidase (CAI; GRIENDLING; HARRISON, 2003; GRIENDLING; FITZGERALD, 2003; CHUNG et al., 2010).

Diferentemente do descrito para o consumo crônico de álcool, as respostas vasculares adaptativas ao estresse em animais e/ou humanos

envolvem: diminuição da reatividade da aorta à noradrenalina (MANUKHINA; LAPSHIN; MEERSON, 1998; CORDELLINI; VASSILIEFF, 1998; NAVARRO-OLIVEIRA; VASSILIEFF; CORDELLINI, 2000), aumento da resposta vascular à acetilcolina (CORDELLINI; VASSILIEFF, 1998; LENASI; STRUCL, 2004), diminuição dos níveis de arginina no plasma (MILAKOFSKY; HARRIS; VOGEL 1993), aumento do RNAm para a óxido nítrico sintase no núcleo paraventricular e córtex da adrenal (CALZÁ; GIARDINO; CECCATELLI, 1993; TSUCHIYA et al., 1997) e aumento da biodisponibilidade de óxido nítrico na circulação renal (DE MORAES et al., 2004). Ainda, as alterações vasculares induzidas pelo estresse mostram-se dependente da integridade do endotélio e da hiperatividade do sistema óxido nítrico endotelial (CORDELLINI; VASSILIEFF, 1998; LANZA JÚNIOR; CORDELLINI, 2007). Contrariamente, Chung et al. (2010) mostraram uma diminuição da resposta vasodilatadora à acetilcolina, mas não ao nitroprussiato de sódio (vasodilatador independente do endotélio). Estes resultados conflitantes mostram que os mecanismos da resposta vascular adaptativa ao estresse ainda estão por ser completamente compreendidos.

Deve-se ressaltar ainda que numa variedade de processos fisiopatológicos, as alterações da função da célula endotelial não estão provavelmente relacionadas à diminuição da produção e/ou liberação de fatores de relaxamento derivados do endotélio, mas à liberação simultânea de fatores de contração derivados do endotélio, por exemplo, produtos da via ciclooxigenase (CORDELLINI, 1999). Embora a taxa de produção de metabólitos da ciclooxigenase em células normais pareça favorecer substâncias vasodilatadoras, é possível que esta taxa possa diferir em

respostas a estados patológicos, por exemplo, estados hipertensivos (CORDELLINI et al., 1990).

Finalmente, a compreensão atual do desenvolvimento das doenças cardiovasculares reconhece o significado de múltiplos fatores de risco diretamente envolvidos na gênese, progressão e ocorrência de eventos cardiovasculares futuros, entre eles, o consumo de etanol e o estresse. Ainda, embora ambos etanol e estresse, isoladamente, desempenhem papel importante no desenvolvimento das doenças cardiovasculares, dúvidas ainda persistem sobre seus efeitos na resposta vascular, especificamente.

Frequentemente, o consumo de etanol acontece no contexto de vários fatores de risco, incluindo a exposição ao estresse, capaz de modificar o impacto do consumo de etanol no organismo. Entretanto, as conseqüências do consumo crônico de etanol, em associação à exposição ao estresse, sobre a função vascular permanecem por ser determinadas. Ainda, embora o consumo de etanol em longo prazo possa afetar as funções cardiovasculares, danos precoces causados pelo consumo de etanol sobre o sistema cardiovascular também precisam ser considerados (RESSTEL et al., 2006).

Pelo exposto, o objetivo do presente estudo foi avaliar, em ratos adultos, o risco cardiovascular do consumo de etanol e da exposição ao estresse, isoladamente e em associação, através da investigação de alterações na pressão arterial e na resposta vascular da aorta torácica a agonistas simpatomiméticos e à angiotensina II, bem como os possíveis mecanismos envolvidos. Essa avaliação foi realizada com foco nas vias de sinalização endotelial do óxido nítrico, da ciclooxigenase e dos receptores α_2, AT_1 e AT_2,

bem como na capacidade antioxidante do plasma e nos niveis plasmáticos de corticosterona.

Capítulos

Endothelial α_2-adrenoceptor and prostanoids on aorta response to stress and ethanol alone or in association

Adrenoceptores alfa-2 e prostanóides endoteliais na resposta da aorta ao stress e etanol, isoladamente ou em associação

Stress and ethanol: α_2-adrenoceptor and prostanoid

Estresse e etanol: adrenoceptor-α_2 e prostanóide

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Palavras-chave: etanol, estresse, reatividade vascular, prostanóides, adrenoceptores alfa-2.

Abstract

Background: stress and ethanol alone are important cardiovascular risk factors.

Objective: to evaluate the vascular risk of ethanol consumption and stress exposure, isolated and in association, in male adult rats.

Methods: rats were separated into control, ethanol (20% in drinking water for 6 weeks), stress (immobilization 1h day/5 days a week during 6 weeks) and stress/ethanol. Curves to noradrenaline, in the absence and presence of yohimbine, L-NAME or indomethacin, and to phenylephrine were obtained in thoracic aortas with (+E) and without (-E) endothelium. EC_{50} and maximum response (n=8-12) were compared by two way ANOVA/Bonferroni.

Results: Stress exposure determined a similar increase in noradrenaline maximum response of intact aortas from stress and stress/ethanol groups while ethanol consumption did not altered the reactivity to noradrenaline (control 2.22±0.12; ethanol 2.35±0.10; stress 2.72±0.11*; stress/ethanol 2.78±0.15*, $P<0.05$ related to control). This hyperreactivity was abolished by endothelium removal and the presence of indomethacin or yohimbine, but was not altered by the presence of L-NAME. None of the protocols changed the EC_{50} values of aortas +E or –E and the maximum response of aortas –E to noradrenaline, as well as the responsiveness to phenylephrine of aortas +E and -E.

Conclusion: stress-induced aorta hyperreactivitity to noradrenaline was shown to be dependent on endothelium integrity and is probably a consequence of endothelium-derived contracting prostanoids released through endothelial alpha-2 adrenoceptor stimulation. The absence of synergistic vascular effects between stress and ethanol suggests that there is a reasonable safety in the association of these cardiovascular risk factors in adult life.

Introduction

Chronic stress is an important risk factor to the development of cardiovascular pathologies[1] such as atherosclerosis[2] endothelial dysfunction[3] and hypertension[4]. However, the severity of the cardiovascular damage caused by stress exposure depends on the nature of the stressor as well as on its intensity and duration[5].

The literature reports that chronic stress impairs the endothelial function, evidenced by a decrease of acetylcholine-induced vasorelaxation[2], as well as a reduction in the aorta nitric oxide (NO) synthase activity[6]. In turn, acute stress exposure reduces noradrenaline-induced contraction and increases acetylcholine-induced relaxation of rat aorta[1,7].

Chronic ethanol consumption may also cause cardiocirculatory modifications that contribute to the development of cardiovascular diseases[8,9]. In this regard, consumption of 20% ethanol during 6 weeks increased the mesenteric vascular bed response to phenylephrine in Wistar rats[8]. Moreover, the chronic consumption of 10% ethanol increased noradrenaline response in aorta from male Sprague-Dawley[10]. The same was observed in mesenteric artery from rats treated with 36% ethanol during 18 weeks[11].

Contrarily, unchanged or decreased phenylephrine responses have been reported in aortas from ethanol exposed rats[12,13]. These discrepancies may reflect differences among the experimental protocols or characteristics of the animal model[14].

Although, ethanol and stress isolated play an important role in the development of cardiovascular disease, doubts still persist about the effects not only of stress but also of ethanol consumption upon the vascular responsiveness. These doubts are even greater in relation to simultaneous exposure to these factors. Thus, the aim of this study was to investigate in adult rats the vascular adaptive response to sympathetic agonists induced by ethanol consumption and exposure to stress, alone or in association, and to extend into its mechanism.

Materials and Methods

Experimental design

Adult male Wistar rats (100 to 120-day old) were housed in plastic cages in a 12 h light-dark cycle at (23±2ºC) and fed with regular lab chow. Along the 6 weeks, these animals were separated into four groups: control, that received water "ad libitum"; stress, that were immobilized in a containment metallic tube (1 hour day/5 days a week) to restrict completely their movements, preserving only the breathing; ethanol, that received ethanol solution at 20% instead of drinking water[15]; stress/ethanol, submitted to immobilization stress and exposed to ethanol (20%). Animals from ethanol and stress/ethanol groups were adapted by increasing concentrations of ethanol consumption (5% in the first week, 10% in the second week and 20% in the third week onward).

All experiments and procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals,* published by the U.S. National Institutes of Health[16] and were approved by the Research Ethics Committee of the Biosciences Institute of Botucatu, University Estadual Paulista.

Blood ethanol

Blood (5 mL per rat) was collected from the aorta of anaesthetized rats using heparinized syringes. Samples (1 mL) were placed in 10mL headspace vials, to which were added 1.0 g sodium chloride, 1.0mL water, 100 mL internal standard (acetonitrile, 1mL L^{-1}). Ethanol analysis was carried out using a CG-17A gas chromatographer (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and an HSS-4A headspace sampler (Shimadzu). Calibration standards (0.10–3.16mg mL^{-1}) were prepared in headspace vials. Ethanol concentrations are expressed in mg mL^{-1} blood.

Aorta rings

Animals were killed in guilhotine. After thoracotomy, the descending thoracic aortas were removed and dissected in 3-4 mm segments (two rings). Indistinctly, one of the rings had his endothelium mechanically removed. The rings were mounted into a 2 mL organ chamber

containing Krebs–Henseleit solution (NaCl 130; KCl 4.7; CaCl2 1.6; KH2PO4 1.2; MgSO4 1.2; NaHCO3 15; glucose 11.1; in mmol/L), fixed to a stainless-steel hook attached to a stationary support as well as to a hook connected to an isometric force transducer. The Krebs–Henseleit solution was kept at pH 7.4 and 37°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Tension was monitored continuously and recorded using a Powerlab 8/30 data-acquisition system (ADInstruments, Castle Hill, NSW, Australia). Prior to the concentration–response curve to agonists, rings were equilibrated for 60 min under a resting tension of 1.5g, which is optimal for inducing the maximum contraction.

The functional state of the endothelium was tested at the beginning of the concentration–response curve by the ability of 10^{-4}M acetylcholine to elicit vasodilator responses in preparations pre-contracted by 10^{-5}M phenylephrine. Preparations that presented more than 80% of relaxation were considered as having an intact endothelium whereas those that showed no relaxation were considered completely devoid of endothelium. Preparations with and without endothelium were studied in parallel.

Organ bath assay

The responses (g) evoked by the addition of noradrenaline (10^{-10} M to 10^{-4} M; Sigma-Aldrich) or phenylephrine (10^{-10} M to 10^{-4} M; Sigma-Aldrich) cumulatively added directly into the organ bath were plotted to obtain concentration–response curve. When appropriated, the noradrenaline responses were also determined in preparations pretreated for 30 min with 10^{-4}M L-NAME (Sigma-Aldrich) or 10^{-5}M indomethacin (Sigma-Aldrich), respective non-selective inhibitors of NOS and cyclooxygenase. In another series of experiments, noradrenaline responses were determined in rings pretreated for 30 min with 10^{-6} M yohimbine (Sigma-Aldrich), and α_2-adrenoceptor antagonist. The inhibitors and the antagonist were added in the last 30-min stabilization period and remained in contact with preparations until the end of the experiment.

Non-linear regression (variable slope) of the obtained concentration-effect curves revealed the R_{max} (maximal response; highest point of each concentration–response curve) and

the EC_{50} (negative logarithm of the concentration that evoked 50% of the maximal response). The EC_{50} is an indicative of sensitivity to the drug studied.

Determination of NO total (NO_2/NO_3)

100µL aliquots of plasma samples from different experimental groups were deproteinated with 200 absolute ethanol and kept for 30 minutes in freezer (-20 ° C). Then, they were subjected to centrifugation (10000rpm for 5 minutes) and the supernatant was collected. For determination of total (NO_2/NO_3) in plasma was used as the reaction medium a mixture of sodium phosphate buffer 20 mM pH 7.4, cofactors (final concentration 100µM NADPH and FAD 5 mM) and nitrate reductase of Aspergillus (Sigma) at a concentration of 0.1 U/mL. Then the samples were incubated for 3 hours in a water bath at 37 ° C and after this period were added to Griess Reagent I (1% sulfanilamide in 5% phosphoric acid) and Griess Reagent II (naftiletilenodiamina 0.1%). Then, there was the reading of compound chromophore in a spectrophotometer at 540 nm. The concentration of the samples were calculated using a standard curve with known concentrations of $NaNO_2$ (0.40 – 200 µl) and expressed in mM/l.

Statistical analysis

The concentration of vasoactive agents producing a response that was 50% of the maximum (EC_{50}) was calculated in each experiment. Data are presented as mean ± SEM. The maximal responses (R_{max}) and EC_{50} values were compared by two-way ANOVA followed by Bonferroni's post-test since one variable was the consumption ethanol and the other, the exposure to immobilization stress. $P < 0.05$ was considered statistically significant.

Results

The noradrenaline responses observed in intact aortas taken from animals exposed to both stress and stress/ethanol, but not from those exposed to ethanol isolated, were higher than those observed in intact aortas from control animals, which resulted in increased values of Rmax (Figure 1A).

The presence of L-NAME increased the noradrenaline Rmax in aortic rings taken from control and ethanol groups permitting responses of similar magnitude with preparations taken

from stress and stress/ethanol groups (Figures 1B). In turn, indomethacin has abolished the elevation of noradrenaline responses in aortas from stress and stress/ethanol groups, thereby resulting in values of Rmax at the level of the control group (Figure 1C). Similarly, yohimbine has also abolished the hyperreactivity to noradrenaline in thoracic aorta taken from stress and stress/ethanol animals (Figure 1D). Moreover, in terms of EC_{50}, no differences were detected among groups either in absence or presence of L-NAME, indomethacin or yoimbine (Table 1).

However, the removal of the endothelium increased the sensitivity to noradrenaline related to the respective aorta with endothelium (Table 1) and abolished the aorta hyperreactivity to NA observed in stress and stress/ethanol groups (Figure 2A). None of protocols altered the reactivity to noradrenaline of denuded aortas (Figure 2B-D) as well as the reactivity to phenylephrine of intact and denuded aortas (Figure 3). Similar phenylephrine EC_{50} were also observed among groups (Table 2).

Blood ethanol concentration reached 0.42 ± 0.09 mg/ml in rats exposed to ethanol during 6 weeks, n=8 and 0.57 ± 0.13 mg/ml in stress/ethanol group, n=10.

The concentration in plasma nitrite/nitrate determined by the Griess Reaction, was not significantly different among groups (Figure 4).

Discussion

Experimental and epidemiological evidences suggest that both ethanol and stress play an important role in the development of cardiovascular diseases[2,17,18]. However, there are discrepancies in the literature about the effects of chronic consumption of ethanol, as well as exposure to stress on the vascular responses to vasoactive agents. Moreover, the literature is scarce regarding the cardiovascular risks of the association of these factors.

The data reported herein have shown that, independently on ethanol consumption, stress determined aorta hyperreactivity to noradrenaline which was dependent on endothelial cell integrity since it was abolished by the removal of the endothelium. On the other hand, chronic ethanol consumption did not influence the noradrenaline responses in rat thoracic aorta. The ethanol consumption also not modified the observed stress-induced elevation of noradrenaline

responses in these preparations. Considering that the absence of endothelium potentiates the contractile response to noradrenaline observed in rats not exposed to stress, the enhanced response to noradrenaline observed in intact aorta from stressed rats could indicate an endothelial dysfunction in this condition.

These findings corroborate previous reports showing that stress promotes endothelial dysfunction[3,19,20,2]. However, other studies reported that stress exposure, rather than impair, may enhance the endothelial function[21,1,22].

The presented data also corroborate studies reporting that consumption of 20% ethanol for 12 weeks did not alter the aorta reactivity to phenylephrine in Fisher rats[13]. Conversely, increased response to noradrenaline was reported in aorta taken from Sprague-Dawley rats exposed to ethanol 10% for 5 months[10]. These differences may be due probably to the characteristics of the different experimental models that were used in these studies. This reinforces the need for further studies in order to have a clearer idea of the real implications of stress and/or chronic ethanol consumption on cardiovascular physiology.

Many studies have shown that changes in the function of endothelial cells are involved in a variety of pathophysiological processes, a phenomenon probably related not to a decrease in the production and/or release of endothelium-derived relaxing factors (EDRFs), but to a simultaneous release of endothelium-derived contracting factors (EDCFs), for example, products of the cyclooxygenase pathway[23].

Although the rate of production of cyclooxygenase metabolites in normal cells appears to facilitate vasodilators, it is possible that this rate may vary in response to disease states, for example, hypertensive states[24].

In order to investigate the hypothesis that EDCFs may be released after exposure to chronic stress, the reactivity to noradrenaline in aorta pretreated with indomethacin was investigated. The presence of this inhibitor abolished the increase of the maximum response to noradrenaline observed in intact aorta of stressed rats. After being restored, this response reached a value similar to that observed in rat aorta with endothelium from controls in the absence of indomethacin. These data suggest that the aorta hyperreactivity to noradrenaline

induced by chronic immobilization stress occurs due to the release of an EDCF, sensitive to indomethacin blockade. EDCF release sensitive to inhibitors of cyclooxygenase have been previously described in micro-and macrovessel of spontaneously hypertensive rats[25] and in aorta of DOCA-salt hypertensive rats[24,23]. Although the present study allows us to conclude that the EDCF released on chronic exposure to immobilization stress is a product of the arachidonic acid metabolism, the identity of this factor remains to be determined.

Once known the participation of EDCF(s) in the stress-induced increase of noradrenaline responses in rat aorta it is pertinent to identify the way in which this sympathetic agonist is able to activate this endothelial mechanism. Previous studies report that α_1 and α_2-adrenorecptores are expressed either in smooth muscle or in endothelial cells[26-28]. Moreover, the stimulation of α_2-adrenoceptor located in the endothelium releases NO[29,30] and/or prostanoids[29] thus counterbalancing the vasoconstrictor effects of noradrenaline mediated by α_1-adrenoceptor present in the smooth muscle layer. It has also shown that thoracic aorta responses to clonidine, a selective α_2-adrenoceptor agonist, may be modulated negatively by NO and positively by vasocontracting prostanoids[31].

In the present study, the presence of yohimbine abolished the stress-induced increase of noradrenaline responses in rat thoracic aorta. Moreover, neither exposure to stress nor ethanol consumption altered the thoracic aorta responses to phenylephrine, a selective α_1-adrenoceptor agonist. These data suggest that the endothelial α_2-adrenoceptor stimulation may release vasoconstrictor prostanoids, increasing the noradrenaline responses in aortas from stress exposed rats.

It is not entirely clear whether only vasoconstrictor prostanoids are responsible to the increased response to noradrenaline in aorta of stressed animals or if they act in parallel with L-arginine/NO mechanisms. The literature reports a simultaneous release of EDRF and EDCF in aorta of DOCA-salt hypertensive rats[32, 24]. Moreover, this increased EDRF release would be a compensatory mechanism for the EDCF release associated with the hypereactivity to noradrenaline and the hyporeactivity to acetylcholine observed in aorta from DOCA-salt hypertensive rats[24,23].

The literature reports a simultaneous release of EDRF and EDCF in aorta of DOCA-salt hypertensive rats[32,24]. Moreover, this increased EDRF release would be a compensatory mechanism for the EDCF release associated with the hypereactivity to NA and the hyporeactivity to ACh observed in aorta from DOCA-salt hypertensive rats[24,25]. Considering that, it was investigated the role of L-arginine/NO pathway in the noradrenaline response of aorta from chronic stressed rats associated or not to ethanol consumption. The results show that the inhibition of NOS by L-NAME increased the noradrenaline Rmax in thoracic aortas taken from either control or ethanol animals without altering the EC50 values, permitting responses of similar magnitude with preparations taken from stress and stress/ethanol groups, in the absence or presence of L-NAME. These data suggest there is no change in the aorta NO-pathway induced by exposure to stress and chronic ethanol consumption, alone or in combination. This hypothesis is also supported by the observation of similar plasma concentration of nitrite/nitrate among the different experimental groups.

In summary, the present study showed that the regime of chronic ethanol consumption employed did not represent a vascular risk factor for adult rats. However, chronic exposure to stress determined an endothelial cell dysfunction which is probably related not to the production and/or release of EDRF/NO but to a simultaneous release of EDCF, sensitive to indomethacin blockade, through α_2-adrenoceptor stimulation. Finally, the absence of vascular synergistic effects between stress and ethanol suggests that there is a reasonable safety in the association of these factors with regard to cardiovascular risk, considering the adulthood.

Acknowledgments

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Tables

Table 1
Values of EC50 to noradrenaline obtained in aortic rings with and without endothelium, in presence of absence of L-NAME, indomethacin or yohimbine from adult male rats exposed or not to chronic stress and ethanol, alone or in combination

	CONTROL EC50 ($x10^{-7}$ M)	STRESS EC50 ($x10^{-7}$ M)	ETHANOL EC50 ($x10^{-7}$ M)	STRESS/ETHANOL EC50 ($x10^{-7}$ M)
With endothelium				
Saline	3.97± 0.06 (10)	3.85 ± 0.04 (10)	4.88 ± 0.06 (10)	4.29 ± 0.08 (10)
L-NAME (10^{-4} M)	2.48 ± 0.05 (09)	2.36 ± 0.05 (09)	2.60 ± 0.04 (09)	2.64 ± 0.08 (09)
Indomethacin (10^{-5} M)	3.85 ± 0.05 (08)	3.56 ± 0.06 (08)	5.39 ± 0.05 (08)	3.58 ± 0.06 (08)
Yohimbine (10^{-6} M)	10.76 ± 0.06 (09)	10.19 ± 0.06 (09)	10.23 ± 0.04 (09)	9.00 ± 0.06 (09)
Without endothelium				
Saline	0.61 ± 0.10* (10)	0.83 ± 0.07* (10)	0.64 ± 0.06 (10)	0.67 ± 0.09* (10)
L-NAME (10^{-4} M)	0.77 ± 0.06* (09)	1.18 ± 0.11* (09)	1.10 ± 0.07* (09)	0.90 ± 0.06* (09)
Indomethacin (10^{-5} M)	0.83 ± 0.05* (08)	1.88 ± 0.06* (08)	1.47 ± 0.05* (08)	1.30 ± 0.08* (08)
Yohimbine (10^{-6} M)	1.82 ± 0.05* (09)	1.94 ± 0.08* (09)	2.10 ± 0.05* (09)	2.75 ± 0.07* (09)

Control: received water "ad libitum"; ethanol: received ethanol solution 20% instead of drinking water; stress: immobilization in a containment metallic tube (1 hour day/5 days a week during 6 weeks); chronic stress/ethanol: submitted to immobilization stress and exposed to 20% ethanol. Data express as mean ± SEM and, between parentheses, the number of independent determinations. *P < 0.05 related to the respective aorta with endothelium.

Table 2
Values of EC50 to phenylephrine obtained in aortic rings with and without endothelium from adult male rats exposed or not to chronic stress or ethanol, alone or in combination

	CONTROL EC50 ($x10^{-7}$ M)	STRESS EC50 ($x10^{-7}$ M)	ETHANOL EC50 ($x10^{-7}$ M)	STRESS/ETHANOL EC50 ($x10^{-7}$ M)
With endothelium	1.58 ± 0.15 (09)	2.25 ± 0.12 (09)	1.14 ± 0.10 (09)	1.79 ± 0.10 (09)
Without endothelium	0.88 ± 0.09* (09)	0.67 ± 0.08* (09)	0.80 ± 0.04* (09)	1.10 ± 0.07* (09)

Control: received water "ad libitum"; ethanol: received ethanol solution 20% instead of drinking water; stress: immobilization in a containment metallic tube (1 hour day/5 days a week during 6 weeks); chronic stress/ethanol: submitted to immobilization stress and exposed to 20% ethanol. Data express as mean ± SEM and, between parentheses, the number of independent determinations. *P < 0.05 related to the respective aorta with endothelium.

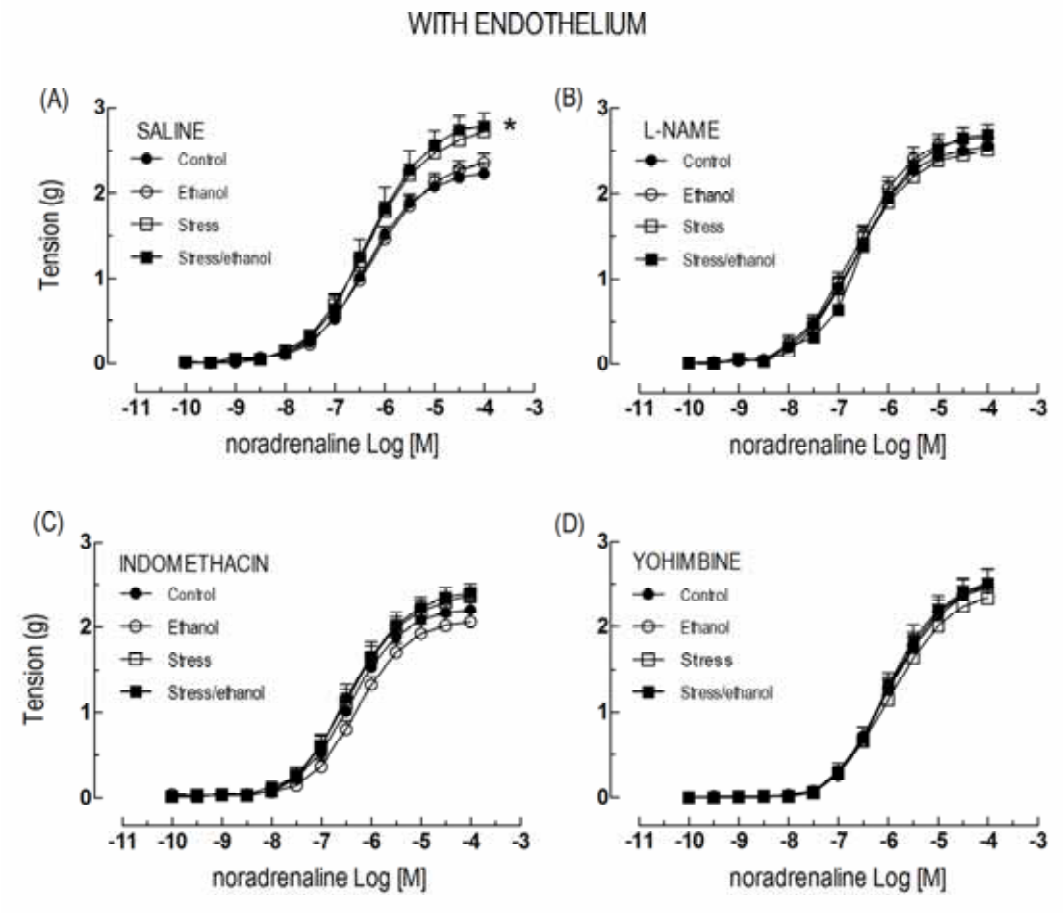

FIGURE 1

Figure 1. Concentration-response curves to noradrenaline obtained in intact thoracic aorta rings taken from animals exposed to ethanol consumption and/or stress, in the absence or presence of L-NAME (10^{-4} M), indomethacin (10^{-5} M) or yohimbine (10^{-6} M). Values are means ± SEM. The number of independent determinations was 8-10.

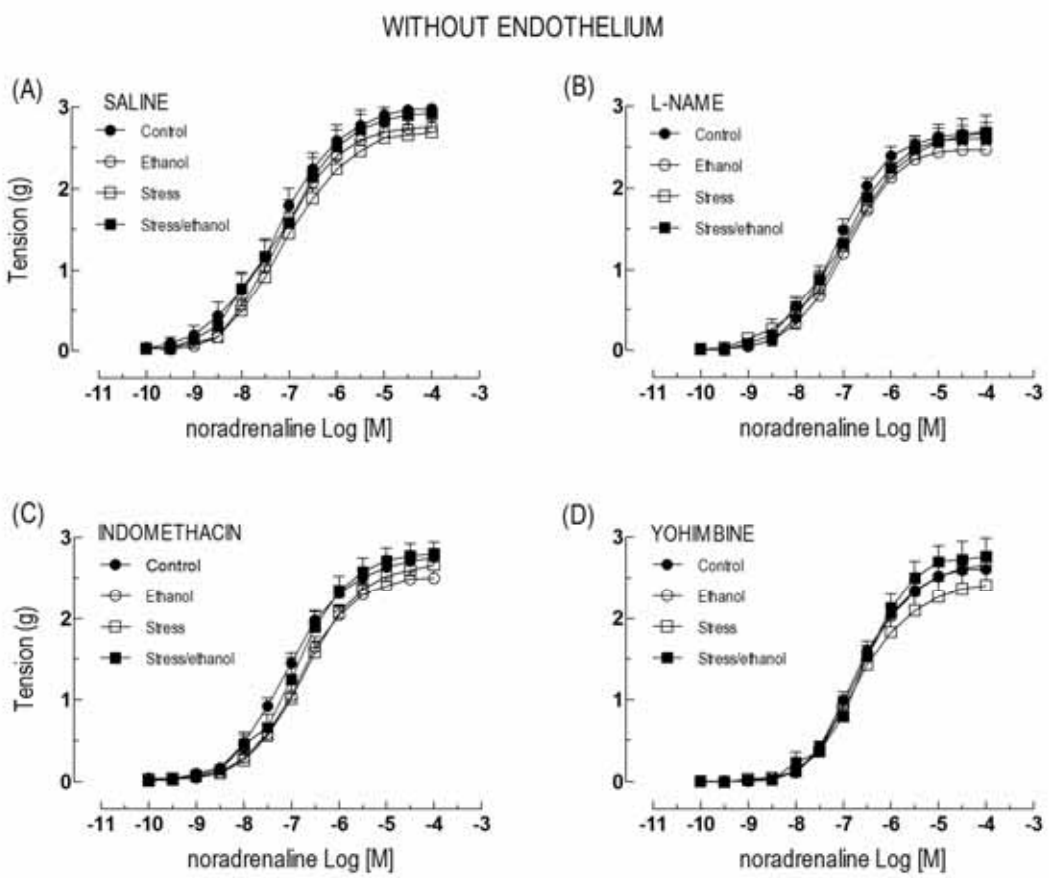

FIGURE 2

Figure 2. Concentration-response curves to noradrenaline obtained in denuded thoracic aorta rings taken from animals exposed to ethanol consumption and/or stress, in the absence or presence of L-NAME (10^{-4} M), indomethacin (10^{-5} M) or yohimbine (10^{-6} M). Values are means ± SEM. The number of independent determinations was 8-10.

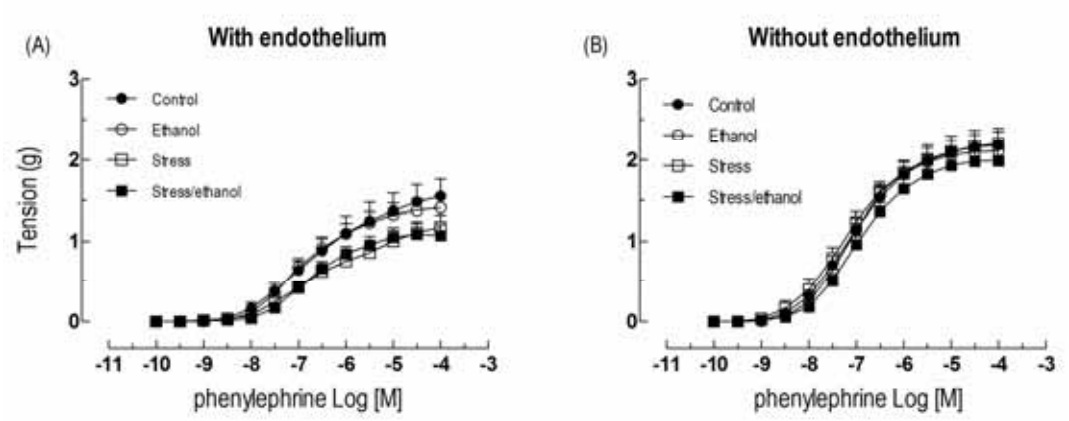

FIGURE 3

Figure 3. Concentration-response curves to phenylephrine obtained in rings of thoracic aorta with and without endothelium taken from animals exposed to ethanol consumption and/or stress. Values are means ± SEM. The number of independent determinations was 8-10.

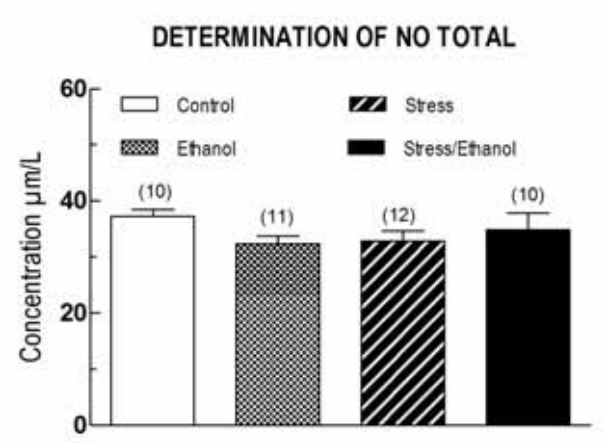

FIGURE 4

Figure 4. Concentration in plasma nitrite/nitrate determined by the Griess Reaction taken from animals exposed to ethanol consumption and/or stress. Values are means ± SEM. The number of independent determinations was 10-12.

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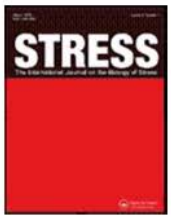
Stress



Endothelial AT1 and AT2 pathways in aortic responses to angiotensin II after stress and ethanol consumption in rats

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Endothelial AT_1 and AT_2 pathways in aortic responses to angiotensin II after stress and ethanol consumption in rats

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Running title: Stress and ethanol: aorta AT signaling

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Abstract

Stress and ethanol are important cardiovascular risk factors. Their vascular and blood pressure (BP) effects were evaluated alone and in combination. Adult male Wistar rats (8-10 per group) were separated into: control, ethanol (ethanol 20% in drinking water for 6 weeks), stress (restraint 1h/day 5 days/week for 6 weeks) and ethanol/stress (in combination) groups. Systolic BP was evaluated weekly. Concentration-response curves for contractile responses to angiotensin II in absence and presence of losartan (AT1-blocker), PD123-319 (AT_2-blocker), L-NAME (nitric oxide synthase inhibitor), or indomethacin (cyclooxygenase inhibitor) were obtained in isolated intact and endothelium-denuded aortas. Effective concentration 50% (EC50) and maximum response (MR) were compared among groups using MANOVA/Tukey tests.. Stress and stress plus ethanol increased BP. Ethanol and stress, alone and in combination, did not alter angiotensin-responses of intact aortas. PD123-319 decreased MR to angiotensin II in intact aortas from the ethanol and ethanol/stress groups relative to control in presence of PD123-319. Losartan increased MR to angiotensin II in intact aortas from the stress and ethanol/stress groups relative to control in presence of losartan. None of the protocols altered angiotensin-responses of denuded aortas. Neither indomethacin nor L-NAME altered angiotensin-responses of intact aortas from the experimental groups. Thus ethanol and ethanol plus stress may alter endothelial signaling via AT_1-receptors, without changing systemic BP. Stress and stress plus ethanol may alter endothelial signaling via AT_2-receptors, and thereby increase BP. Knowledge of such vascular changes induced by stress and/or ethanol, may contribute to understanding adverse cardiovascular effects of stress and ethanol consumption in humans.

Introduction

Chronic stress is an important risk factor for the development of cardiovascular pathologies such as atherosclerosis and arterial hypertension (Chung et al., 2010; Cordellini and Vassilieff, 1998; Diaconu et al., 2011; Ghiadoni et al., 2000). Moreover, the severity of the cardiovascular damage caused by stress exposure depends on the nature of the stressor, as well as on its intensity and duration (Hjemdahl, 2002). The literature reports that chronic stress impairs endothelial function, as evidenced by the increase of angiotensin-induced vasoconstriction in the aorta due to reduced endothelial nitric oxide (NO) buffering capacity via the angiotensin/AT_2 receptor pathway (Loria et al., 2011). Oxidative stress, via activation of the angiotensin/AT_1 receptor pathway, has also been shown to impair endothelial function (Chung et al. 2010).

Similarly, ethanol consumption may cause cardiocirculatory alterations that contribute to the development of cardiovascular diseases (Tirapelli et al., 2007, 2008). In this regard, chronic ethanol consumption induces activation of the renin-angiotensin system (Wakabayashi and Hatake, 2001). Moreover, the arterial hypertension reported in this condition seems to be correlated to activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase causing endothelial injury and impairment of the endothelial NO generating system (Husain et al., 2005, 2008, 2010).

Our current understanding of the development of cardiovascular diseases recognizes the significance of multiple risk factors; which have been shown to be directly implicated in the genesis, progression and occurrence of future cardiovascular events. Two of these cardiovascular risk factors are ethanol consumption and stress exposure. While many studies examine the effects of ethanol consumption alone, this condition usually happens in the context of various risk factors, including stress exposure able to modify the impact of ethanol consumption on the body. However, the consequences of chronic ethanol consumption in association with stress exposure on vascular function remain to be determined. Moreover, the literature has shown that although long-term ethanol consumption can affect cardiovascular functions, early damage caused by ethanol consumption on the cardiovascular system also needs to be considered (Resstel et al., 2006).

Thus, the purpose of the present study was to evaluate the cardiovascular risk of stress

exposure and ethanol consumption, alone and in combination, through the investigation of possible changes in arterial blood pressure and vascular reactivity to angiotensin II, assessed in vitro. A second purpose was to evaluate in detail the mechanisms underlying the effects of long-term ethanol consumption and stress exposure, alone and in combination, on angiotensin-induced contraction of the aorta. This evaluation was conducted with a focus on NO, cyclooxygenase and endothelial AT_1/AT_2-signaling pathways, as well as plasma antioxidant capacity.

Methods

Experimental Design

Experiments were performed on adult male Wistar rats (90 to 120-day old) obtained from UNESP - Univ Estadual Paulista facilities. They were housed in individual plastic cages under a 12 hour light-dark cycle (lights on/off at 07:00/19:00 h) at 23 ± 2ºC and fed with regular lab chow. The rats were separated into four groups (8-10 rats per group): control (received water *ad libitum*); stress, (restraint for 1 hour/day 5 days/week for 6 weeks); ethanol, [20% (v/v) ethanol solution instead of tap water for 6 weeks (Tirapelli et al., 2006)]; ethanol/stress (20% ethanol solution and restraint stress for 6 weeks). Rats from the ethanol and ethanol/stress groups were adapted to ethanol consumption by gradually increasing the concentration of ethanol in their drinking water (5% in the first week, 10% in the second week, 20% in the third week and 20% for 6 additional weeks).

Rats were restrained, but not immobilized, in a cylindrical metallic tube measuring 27.0 cm long × 6.1 cm internal diameter containing ventilation holes, individually adapted to completely restrict movements, preserving only their breathing. A slotted opening allowed for free mobility of the tail. Exposure to this stressful stimulus took place between 08:00h and 10:00h. Restraint is a well validated method to induce stress (Buynitsky and Mostofsky, 2009). The duration of the stressor used, *i.e.*, the length of time of exposure during each session was based on the work of Marin et al. (2007).

During the stress sessions, rats from the control and ethanol groups remained in their cages. Twenty-four hours after the last stress session, the rats were killed by decapitation, without

anaesthesia, and the aorta removed for the different experimental protocols.

Animal procedures were in accordance with the principles and guidelines of the National Council of Control of Animal Experimentation and the study was approved by the Ethical Committee for Animal Experimentation (protocol number: 295-CEEA).

Measurement of Blood Pressure

Systolic blood pressure (mmHg) was determined weekly during the adaptation and exposure periods. Measurements were taken from conscious rats using the tail-cuff plethysmographic method (Narco Bio-Systems, Inc., Houston, TX). The rats were pre-warmed in a Bio-Heater (Insight Ltda. Ribeirão Preto - Brazil) at ~40°C for 5-10 min and then placed into a restrainer tube for blood pressure measurement. Three consecutive recordings (~1 min apart) were performed, and the mean of these three measurements was recorded.

Vascular Reactivity Protocol

Immediately after the rat had been decapitated, the descending thoracic aorta was excised and trimmed free of adhering fat and connective tissue. Two transverse rings of the aorta, each about 4 mm in length were cut. One ring served as control (intact aorta), while the endothelium was mechanically removed from the other by gently rubbing the luminal surface (denuded aorta).The rings were mounted into a 2 ml organ chamber containing Krebs–Henseleit solution, with the composition (in mM): NaCl 130.0; KCl 4.7; $CaCl_2$ 1.6; KH_2PO4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 15.0; glucose 11.1. The Krebs–Henseleit solution was kept at pH 7.4 and 37°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Tension was monitored continuously and recorded using a Powerlab 8/30 data-acquisition system (AD Instruments, Castle Hill, NSW, Australia). Prior to the collection of contractile responses to increasing agonist concentrations, to construct concentration-response curves, the rings were equilibrated for 60 min under a resting tension of 1.5 g, which is optimal for inducing a maximum response. All drugs were obtained from Sigma Chemical Co., St Louis, Missouri, USA. They were

dissolved in Krebs-Henseleit solution and the concentrations expressed in molarity.

The functional state of the endothelium was tested at the beginning of the concentration–response curve measurements by the ability of 10^{-4} M acetylcholine to elicit vasodilator responses in preparations pre-contracted by 10^{-5} M phenylephrine. Preparations that presented more than 80% of relaxation were considered as having an intact endothelium, whereas those that showed no relaxation were considered completely devoid of endothelium. Preparations with and without endothelium were studied in parallel.

Angiotensin II (10^{-10}-10^{-6} M) was cumulatively added to the organ bath, and the evoked responses (g of tension) were plotted to obtain the cumulative concentration–response curves. At the end of obtaining these curves, a single dose of sodium nitroprusside (10^{-4} M) was used to test the integrity of the smooth muscle layer.

Involvement of endothelial AT_1/AT_2-signaling pathway

Cumulative concentration-response curves to angiotensin II were also obtained in intact and denuded aortas in the presence of losartan (10^{-7} M, specific AT_1-receptor blocker) or PD123-319 (10^{-7} M, specific AT_2-receptor blocker) introduced into the organ bath 20 minutes before starting measurement of angiotensin II responses.

Involvement of NO and Cylooxygenase Pathways

In another series of experiments, angiotensin II responses were obtained in intact aortas pretreated with L-NG-nitroarginine methyl ester (L-NAME; 10^{-4} M, nonselective nitric oxide synthase inhibitor) or indomethacin (10^{-5} M, non selective cyclooxygenase inhibitor) added to the organ bath 20 minutes before measuring angiotensin II responses.

Plasma Corticosterone Concentration

Immediately after the rat had been decapitated, trunk blood was collected in plastic tubes containing heparin solution 5000 IU/ml (10 µl/ml blood). The whole blood was centrifuged at 3000 *g* for 20 min. at 4°C and plasma was separated for later extraction. Plasma corticosterone

concentrations, a classic index for a stressful paradigm, were determined by use of the Vecsei (1979) method, modified by the previous extraction of the steroid with 1 ml of ethanol. For the assay, the anti-corticosterone antibody, AB-cort-17984, was used. The antibody was sourced from rabbit, with the hormone conjugated to bovine albumin. The specificity of the antibody was tested for cross-reactivity with cortisol (1%), 17-OH-progesterone (1%) and dehydroepiandrosterone sulfate (12%). Tritium-labeled corticosterone was used as the labeled hormone and the separation of the bound and free fraction was carried out with a solution of charcoal/dextran 0.5/0.05%. The minimum detectable concentration was 0.4 µg/dl. The coefficients of intra- and inter-assay variation were 2.8% and 17.8%, respectively.

Plasma Antioxidant Capacity

Ferric reducing ability of plasma (FRAP) measurements were made using a slightly modified version of the methods outlined by Benzie and Strain (1996).

The FRAP technique is based on the ability of plasma to reduce Fe^{+++} ions to Fe^{++} in the presence of 2,4,6 tripyridyl-s-triazine at low pH, forming an Fe^{++} complex tripyridyl-s-triazine; the blue coloring is measured as change of absorbance at 593 nm. The working reagent was prepared by adding 25 ml of acetate buffer pH 3.6 and 2.5 ml 2,4,6 tripyridyl-s-triazine 10 mM to 40 mM HCl and 2.5 ml of $FeCl_3.6H_2O$ 20 mM. For measurements, 300 µl of working reagent was placed in a cuvette and incubated in a water bath at 37°C for 5 minutes. Next, 10 µl of plasma were added, followed by 30 µl of H_2O. The solution was then incubated in a water bath at 37°C for exactly 15 minutes. Subsequently, changes in optical density were recorded in a spectrophotometer (Quimis) against a reagent blank. The values of the antioxidant capacity of plasma were calculated by comparing the absorbance with the absorbance of Fe^{++} solutions of known concentrations (100-1000 mM), which were tested in parallel.

Data Analysis and Statistics

The concentration of angiotensin II producing a response that was 50% of the maximum (EC50) was calculated in each experiment. Data are presented as mean ± SEM or SD. The maximal responses and EC50 values were compared by two-way ANOVA followed by Tukey's post-test (SigmaStat 3.2). The factors in the analysis were ethanol consumption and stress exposure. $P < 0.05$ was considered statistically significant. If necessary, variables were log transformed prior to parametric testing.

Results

Blood Pressure

Exposure to stress, alone and in combination with ethanol, resulted in an increase in the blood pressure in the last week of exposure (6^{th} week) when compared to the control group. Contrarily, no change in blood pressure was observed after chronic ethanol consumption alone (Figure 1).

Aorta Reactivity to Angiotensin II

In the absence of receptor blockers, the vasoconstriction induced by angiotensin II in intact and denuded aortas was not altered by any of the experimental protocols, *i.e.*, chronic ethanol consumption and stress exposure alone and in combination (Table 1).

Involvement of endothelial AT_1/AT_2-signaling pathway

In intact aortas taken from the control and ethanol groups, the presence of the AT_1-receptor blocker, losartan, determined a similar decrease in the maximum response to angiotensin II compared to the same preparations in the absence of blocker (Table 1). However, the presence of this antagonist did not cause any change in the reactivity to angiotensin II in intact aortas from rats submitted to stress, -whether alone or in combination, compared to the same preparations in the absence of blocker (Table 1).

The presence of the AT_2-receptor blocker, PD123-319, caused a similar decrease in the

maximum response to angiotensin II in intact aortas from ethanol and ethanol/stress groups compared to the same preparations in the absence of blocker (Table 1). However, the presence of this antagonist did not cause any change in the reactivity to angiotensin II in the intact aortas of rats from the control and stress groups compared to the same preparations in the absence of blocker (Table 1).

In the absence of blockers, the EC50 values for angiotensin II in aortas did not differ among different experimental groups, either in the absence or presence of endothelium (Table 1). The presence of losartan, but not PD123-319, increased the EC50 values for angiotensin II in intact and denuded aortas, with similar values in all experimental groups (Table 1).

Removal of the endothelium increased the maximum response to angiotensin II that reached similar values among all experimental groups and protocols, without altering the EC50 values (Table 1).

Involvement of NO and Cyclooxygenase Pathways

The presence of indomethacin did not cause any change in the reactivity to angiotensin II in intact aortas from all experimental groups (Table 2).

The presence of L-NAME determined a similar increase in the reactivity to angiotensin II in intact aortas from all experimental groups (Table 2).

Corticosterone Concentration

The plasma concentration of corticosterone was higher in rats submitted to stress, both alone and in combination with ethanol, but was not altered by ethanol consumption alone [mean ± SEM: control 2.26 ± 0.20 µg/dl; stress 4.03 ± 0.50* µg/dl; ethanol 2.27 ± 0.27 µg/dl; ethanol/stress 4.44 ± 0.53† µg/dl; n = 8-12; *p = 0.0195 and $^{\dagger}p$ = 0.0059 compared to control (two-way ANOVA followed by Tukey's post-test)].

Plasma Antioxidant Capacity

No statistically significant alteration in plasma antioxidant capacity was observed in any of the

experimental groups [mean ± SEM: control 762.6 ± 37.4 µmol/l Fe^{+2}; stress 817.6 ± 50.0 µmol/l Fe^{+2}; ethanol 736.2 ± 31.9 µmol/l Fe^{+2}; ethanol/stress 686.3± 38.0 µmol/l Fe^{+2}; n = 10-12; $p > 0.05$ compared to control (two-way ANOVA followed by Tukey's post-test)].

Discussion

The present study demonstrated that chronic exposure to restraint stress, regardless of ethanol consumption, increased blood pressure and impaired the aortic endothelial AT_2-pathway that mediates endothelium-dependent relaxation of smooth muscle to angiotensin II. Conversely, chronic ethanol consumption, regardless of exposure to stress, impaired the endothelial AT_1-pathway that mediates endothelium-dependent constriction of smooth muscle to angiotensin II, but did not alter blood pressure.

Experimental and epidemiological evidence suggests that both stress and ethanol play an important role in the development of cardiovascular disease (Chung et al., 2010; Husain et al., 2010; Rozanski et al., 2005). However, there are discrepancies in the literature regarding the effects of ethanol consumption and stress exposure on regulatory cardiovascular responses.

Low concentrations of ethanol seem to be beneficial to endothelial function and therefore may decrease blood pressure (Jones et al., 2013; Toda and Ayajiki, 2010). However, high doses of ethanol or its chronic consumption can determine a loss in function and impaired viability of endothelial cells (Toda and Ayajiki, 2010) as well as significant changes in the heart and circulatory functions. These findings have identified high/chronic ethanol consumption as a significant risk factor in the development of cardiovascular diseases such as arterial hypertension (Husain et al., 2005, 2008; Sun and Mayhan, 2001; Tirapelli et al., 2007, 2008a). However, Hatton et al. (1992) and Kleinhenz et al. (2008) reported a decrease in blood pressure of rats subjected to chronic ethanol consumption. In the present study, however, no change in blood pressure was observed amongst rats submitted to chronic ethanol consumption alone.

In men, alcohol consumption has been categorized as light (0.1 to 9.9 g/d, or <1 drink daily), moderate (10.0 to 29.9 g/d, or 1 to 2 drinks daily), and heavier (≥ 30.0 g/d, or ≥ 3 drinks daily) (Mukamal et al., 2005). In the present study, the use of 20% ethanol corresponded to a daily intake of 6.0 to 8.0 g. Thus, the chronic ethanol consumption protocol employed in this study might be considered a light daily intake of alcohol, which could partially explain the lack of alteration in blood pressure observed after this protocol alone.

The literature is also not in agreement in regards to cardiovascular adaptive responses induced by stress exposure. Increase or decrease in blood pressure depends on the stressor and the duration and/or frequency of stress exposure (Chung et al., 2010; Matthews et al., 2001; Sparrow et al., 1987). In the present study, chronic exposure to restraint stress alone and in combination with ethanol consumption resulted in hypertension in the last week of exposure.

The most common responses to stress involve activation of the sympathetic nervous system, hypothalamic-pituitary-adrenal axis and renin-angiotensin system (Hjemdahl, 2002; McEwen et al., 1998; Viau and Meaney, 1991). As a consequence, elevations in serum corticosterone and catecholamine concentrations can be seen after stress exposure (Pacak and Palkovits, 2001). Corroborating the literature, the chronic exposure to stress alone and in combination with ethanol consumption caused a significant increase in plasma corticosterone concentration. As the chronic stress state has been defined as persistent visceral arousal coupled with behavioral abnormalities (Ottenweller et al., 1992), the increase in corticosterone concentration partially meets the assumption of the chronic stress paradigm and thus validates our procedure. Contrarily, chronic ethanol consumption did not alter the plasma corticosterone concentration suggesting that this protocol did not represent a stressogenic condition.

Both stress and chronic ethanol consumption induce activation of the renin-angiotensin-system. This endocrine system has angiotensin II as one of its main effectors whose effects are experienced by interacting with the specific receptors, subtype-1 (AT_1) and subtype-2 (AT_2) (Leung, 2004; Wakabayashi and Hatake, 2001).

Angiotensin II induces constriction of the vascular wall by a direct action on smooth muscle

cells, but this effect might be modulated by the action of angiotensin II on endothelial cells. Similar to smooth muscle, the endothelial cells present AT_1 and AT_2 receptors that are involved in the release of endothelium-derived relaxing and/or contracting factors (Pueyo and Michel, 1997). The relative predominance of one or other of the AT-receptor pathways may determine the final action of angiotensin II in these cells.

Normally, the contractile response overcomes relaxation. In several vascular beds, activation of AT_1- and AT_2-receptors leads to antagonistic effects, promoting a counterbalance in vasoactive responses (Fyhrquist and Saijonmaa, 2008). This is evident in the aorta of rats that have had the gene for the AT_2-receptor deleted, where the contractile response to angiotensin II was higher compared to the control group (Akishita et al., 1999). Moreover, the blockade of AT_1-receptors lowers while the AT_2 blockade increases blood pressure in rats, indicating again a counterbalance in response to angiotensin II (Munzenmaier and Greene, 1996). Thus, AT_2 signaling is antagonistic to AT_1 signaling, and results in bradykinin and NO formation (Rush and Aultman, 2008). However, AT_1 signaling results in increased superoxide production via NADH/NADPH oxidase activation and enhanced generation of endothelium-derived constricting factors, such as endothelin-1 (Toda et al., 2007; MacKenzie, 2011). In this context, it should be considered that the AT_1-receptor blockade leaves uncovered the endothelial signaling pathway through AT_2-receptors that becomes a brake for the constriction caused by angiotensin II acting directly on the smooth muscle cell. Furthermore, the blockade of AT_2-receptors leaves uncovered the endothelial signaling pathway through AT_1-receptors that increases the vasoconstriction by angiotensin II acting directly on the smooth muscle cells. Corroborating these findings, angiotensin II-induced vasoconstriction was potentiated by endothelium removal and L-NAME presence, regardless of the protocols of exposure.

It is noteworthy that the increase in blood pressure of rats chronically treated with ethanol is related to increased angiotensin II concentration in plasma and aorta (Husain et al., 2008). Angiotensin II is also responsible for the amplification of the actions of the sympathetic nervous system (Kawasaki et al., 1984; Malik and Nasjletti, 1976). Moreover, chronic angiotensin II signaling induces vascular dysfunction, however pharmacological management of the renin-angiotensin system

can not only control blood pressure, but also correct endothelial dysfunction (Rush and Aultman, 2008). In this context, the effects of chronic ethanol consumption and stress exposure - alone and in combination - were investigated. This investigation was conducted through analysis of vasomotor responses of aorta to angiotensin II in the absence and presence of losartan, PD123-319, indomethacin, or L-NAME.

Neither ethanol consumption nor stress exposure, alone or in combination, caused any change in aortic responses to angiotensin II. Nevertheless, previous studies from our laboratory have shown that the decrease in the vascular reserves determined by the presence of inhibitors such as L-NAME and indomethacin may unveil vascular changes that are below the level of security of vascular function (Grizzo and Cordellini, 2008). In this context, the final responses to angiotensin II in aortas of rats submitted to stress and/or ethanol intake may vary considering the changes in the endothelial cell output determined by the presence of AT-receptor blockers, L-NAME or indomethacin. However, in the present study, presence of neither inhibitors nor blockers unveiled any change in vascular reactivity to angiotensin II in denuded aortas of adult rats exposed to stress and ethanol consumption, alone and in combination.

In contrast, in intact aorta, the presence of losartan showed an impairment of AT_2-receptor endothelial signaling induced by chronic exposure to stress, which was seen regardless of ethanol consumption. The impairment of this signaling was inferred from the observation that the presence of losartan resulted in similar degrees of hyperreactivity to angiotensin II in intact aortas from the stress and stress/ethanol groups when compared to the control group in the same condition, *i.e.*, presence of losartan. Moreover, this difference was abolished by removal of the endothelium. Conversely, the presence of PD123-319 did not show any stress-induced alteration in endothelial signaling via AT_1-receptors, as the aortic reactivity to angiotensin II was similar in the control and stress groups in the presence of this blocker. These data indicate a decrease in the bioavailability of endothelial-derived relaxing factors via AT_2-receptor as a result of stress exposure, alone and in combination with ethanol. Moreover, the change in the endothelial cell output could be responsible for the etiology and/or maintenance of the hypertensive state observed in the stress condition. Taken in conjunction,

these findings raise concern about the higher cardiovascular vulnerability of stress-exposed individuals in the presence of associated pathologies that impair the endothelial AT_2-signaling pathway.

In addition, the presence of PD123-319 showed an impairment of AT_1-receptor endothelial signaling induced by chronic ethanol consumption, alone and in combination with stress. The impairment of this signaling was inferred from the observation that the presence of PD123-319 resulted in similar degrees of hyporeactivity to angiotensin II in intact aortas from the ethanol and ethanol/stress groups when compared to the control group in the same condition, *i.e.*, presence of PD123-319. Moreover, this difference was abolished by removal of the endothelium. Conversely, the presence of losartan did not show any ethanol-induced alteration in endothelial signaling via AT_2-receptors, as the aortic reactivity to angiotensin II was similar in the control and ethanol groups in the presence of this blocker. These data indicate that ethanol consumption, alone and in combination with stress, induces a decreased bioavailability of endothelium-derived contracting factors via AT_1-receptors.This beneficial effect might be partially responsible for there being no change in blood pressure amongst rats submitted to chronic ethanol consumption. Thus, this finding expands upon previous reports showing the beneficial cardiovascular effects of low concentrations of alcohol consumption (Jones et al., 2013; Toda and Ayajiki, 2010).

Although the results show that chronic ethanol in association with stress exposure alters endothelial signaling via AT_1- and AT_2-receptors in intact rat aortas, there was no sign of interaction between these factors. This conclusion was reached based upon the finding that the hyperreactivity to angiotensin in the aortas of stressed rats in the presence of losartan, as well as the hyporeactivity displayed in those subjected to chronic ethanol consumption in the presence of PD123-319, were of similar magnitudes to those observed in rats from the ethanol/stress group in the presence of the same antagonists. Thus, in this study, chronic consumption of ethanol and stress exposure did not constitute additional cardiovascular risk factors, reinforcing the literature reports showing that moderate alcohol consumption might reduce cardiovascular disease (Jones et al., 2013).

The question arises about whether changes in vascular reactivity to stress and ethanol consumption are specific for the angiotensin system. However, previous studies in our laboratory have shown that a similar protocol of stress exposure, alone and in combination with ethanol consumption, increases noradrenaline-induced contraction in rat aorta (Baptista et al., 2014). Moreover, Tirapelli et al. (2008) reported that a similar protocol of ethanol consumption increases phenylephrine-induced contraction, as well as decreases acetylcholine-induced relaxation, in the rat mesenteric arterial bed. It is also important to consider that the vascular reactivity alterations may be blood pressure dependent since the stress exposed rats were hypertensive at the point of the *ex vivo* experiments with the vascular tissue. However, further studies are necessary to clarify this correlation.

The literature has also reported the involvement of cyclooxygenase and/or nitric oxide pathways in changes to vascular reactivity as a result of chronic ethanol consumption and stress exposure (Cordellini et al., 2006; Loria et al., 2011; Tirapelli et al., 2006). In order to investigate the role these pathways may play in the alteration of aortic reactivity to angiotensin II, indomethacin and L-NAME were used. However, in our experiments, the presence of these inhibitors did not change aortic reactivity to angiotensin II in any of the experimental groups. This outcome effectively ruled out the involvement of cyclooxygenase and nitric oxide pathways in the endothelial alterations induced by ethanol consumption and stress exposure alone and in combination. These data also corroborate previous reports from our laboratory showing no change in the plasma concentration of nitrites/nitrates in rats submitted to the same protocols of exposure (Baptista et al., 2014). Similarly, the FRAP method did not show any change in plasma antioxidant capacity throughout all experimental groups, suggesting that the antioxidant system is not involved in the changes in bioavailability of endothelial factors induced by the ethanol and stress conditions. Taking these data into consideration, the identity of the endothelial factors involved in the aortic adaptive response brought on by stress exposure and ethanol consumption alone and in combination requires further investigation.

Conclusions

Chronic ethanol consumption alone and in combination with stress altered endothelial signaling via AT_1-receptors, resulting in aortic hyporeactivity to angiotensin II in the presence of an AT_2-receptor blocker. Ethanol consumption alone resulted in no change in blood pressure. Meanwhile, exposure to stress alone and in combination with ethanol altered endothelial signaling via AT_2-receptors, resulting in aortic hyperreactivity to angiotensin II in the presence of an AT_1-receptor blocker, as well as increased blood pressure. However, the mechanisms involved in these alterations remain to be elucidated. Thus, data presented here may contribute to the body of evidence about disordered cardiovascular function induced by chronic stress and ethanol intake.

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Declaration of Interest

The authors declare no conflict of interest.

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Figure 1

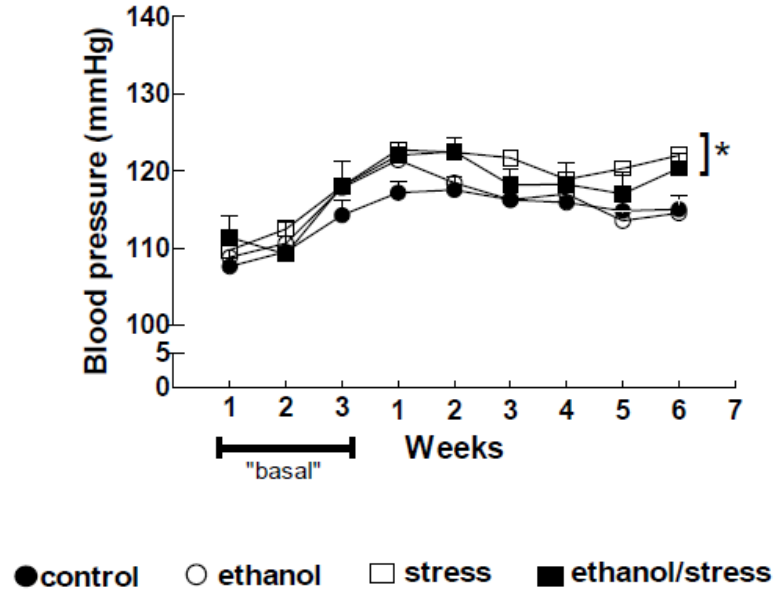

Figure 1. Evolution of caudal systolic blood pressure of adult male rats submitted to chronic ethanol consumption and exposed to stress, alone and in combination. Control - received water *ad libitum*; stress - restraint 1 hour/day 5 days/week for 6 weeks; ethanol - 20% ethanol solution instead of tap water for 6 weeks; ethanol/stress - restraint stress and 20% ethanol solution for 6 weeks. "Basal" - adaptation period for ethanol intake. Values are means ± SEM. Two-way ANOVA followed by Tukey's post-test. (*) $P < 0.05$ relative to control. Number of rats per group = 8-10.

Table 1. Maximum response and EC50 values to angiotensin II in the absence and presence of losartan or PD123-319 obtained in thoracic aorta rings, with and without endothelium, isolated from adult male rats submitted or not to ethanol consumption and restraint stress, alone and in combination

Groups	Maximum response[a] (g of tension)		EC50[b] ($x10^{-8}$ M)	
	With endothelium	Without endothelium	With endothelium	Without endothelium
Absence of blocker				
Control	0.17 ± 0.02^{A}	0.68 ± 0.07^{B}	0.10 ± 0.02^{A}	0.16 ± 0.02^{A}
Ethanol	0.20 ± 0.04^{A}	0.57 ± 0.07^{B}	0.18 ± 0.03^{A}	0.31 ± 0.02^{A}
Stress	0.25 ± 0.03^{A}	0.74 ± 0.07^{B}	0.11 ± 0.02^{A}	0.22 ± 0.02^{A}
Ethanol/Stress	0.20 ± 0.04^{A}	0.50 ± 0.08^{B}	0.10 ± 0.01^{A}	0.15 ± 0.02^{A}
Losartan presence				
Control	0.07 ± 0.01^{C}	0.60 ± 0.09^{B}	6.17 ± 0.26^{B}	9.44 ± 0.16^{B}
Ethanol	0.08 ± 0.01^{C}	0.56 ± 0.11^{B}	5.37 ± 0.37^{B}	6.84 ± 0.20^{B}
Stress	0.22 ± 0.06^{A}	0.66 ± 0.15^{B}	11.9 ± 2.74^{B}	17.52 ± 2.68^{B}
Ethanol/Stress	0.20 ± 0.11^{A}	0.55 ± 0.08^{B}	19.3 ± 2.65^{B}	8.21 ± 0.21^{B}
PD123-319 presence				
Control	0.24 ± 0.06^{A}	0.79 ± 0.08^{B}	2.04 ± 1.14^{A}	0.44 ± 0.02^{A}
Ethanol	0.09 ± 0.02^{C}	0.68 ± 0.07^{B}	0.12 ± 0.05^{A}	0.33 ± 0.02^{A}
Stress	0.18 ± 0.05^{A}	0.84 ± 0.13^{B}	0.45 ± 0.05^{A}	0.42 ± 0.02^{A}
Ethanol/Stress	0.08 ± 0.01^{C}	0.66 ± 0.09^{B}	0.20 ± 0.04^{A}	0.42 ± 0.02^{A}

EC50 – effective concentration 50%. Values are means ± SEM[a] or SD[b]; losartan (10^{-7} M) - AT_1 receptor blocker; PD123-319 (10^{-7} M) – AT_2 receptor blocker; control - received water *ad libitum*; stress - restraint in a metallic tube 1 hour/day 5 days/week for 6 weeks; ethanol - received a 20% ethanol solution instead of tap water for 6 weeks; ethanol/stress - submitted to restraint stress and received a 20% ethanol solution for 6 weeks; inside the same parameter, i.e., maximum response and EC50, mean values sharing a common letter (A, B, or C) were not statistically different ($p > 0.05$, two-way ANOVA followed by Tukey's post-test); number of rats per group = 8-10.

Table 2. Maximum response and EC50 to angiotensin II values in the presence of indomethacin or L-NAME obtained in intact aorta rings isolated from adult male rats submitted or not to ethanol consumption and restraint stress, alone and in combination

Groups	Maximum response[a] (g of tension)		EC50[b] ($x10^{-8}$ M)	
	Indomethacin	L-NAME	Indomethacin	L-NAME
Control	0.34 ± 0.04	0.47 ± 0.05	0.16 ± 0.02	0.19 ± 0.02
Ethanol	0.26 ± 0.06	0.47 ± 0.07	0.22 ± 0.03	0.12 ± 0.02
Stress	0.33 ± 0.06	0.56 ± 0.09	0.15 ± 0.02	0.11 ± 0.02
Ethanol/Stress	0.24 ± 0.03	0.48 ± 0.08	0.17 ± 0.02	0.20 ± 0.02

EC50 – effective concentration 50%. Values are means ± SEM[a] or SD[b]; indomethacin (10^{-5} M) – cyclooxygenase inhibitor; L-NAME (10^{-4} M) – nitric oxide synthase inhibitor; control - received water *ad libitum*; stress - restraint in a metallic tube 1 hour/day 5 days/week for 6 weeks; ethanol - received a 20% ethanol solution instead of tap water for 6 weeks; ethanol/stress - submitted to restraint stress and received a 20% ethanol solution for 6 weeks; two-way ANOVA ($P > 0.05$); number of rats per group = 8-10.

Conclusões

- A hiperreatividade à noradrenalina induzida pela exposição ao estresse, isoladamente e em associação, mostrou-se dependente da integridade do endotélio e envolve a liberação de prostanóides vasoconstritores via estimulação de adrenoceptores alfa-2 endoteliais.
- O consumo de etanol, isoladamente e em associação, alterou a sinalização endotelial via receptores-AT_1, sem alterar a pressão arterial sistólica caudal.
- A exposição ao estresse, isoladamente e em associação, alterou a sinalização endotelial via receptores-AT_2, e, paralelamente, aumentou a pressão arterial sistólica caudal.

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