Hypochlorous Acid Inhibition by Acetoacetate: Implications on Neutrophil Functions

Miriani da Costa, Valdecir Farias Ximenes, and Luiz Marcos da Fonseca*

Department of Clinical Analysis (LAC), State University of São Paulo (UNESP), School of Pharmaceutical Science; Araraquara, SP, CEP 14801–902, Brazil. Received January 19, 2004; accepted April 9, 2004

Type-1 diabetic patients experience hyperketonemia caused by an increase in fatty acid metabolism. Thus, the aim of this study was to measure the effect of ketone bodies as suppressors of oxidizing species produced by stimulated neutrophils. Both acetoacetate and 3-hydroxybutyrate have suppressive effect on the respiratory burst measured by luminol-enhanced chemiluminescence. Through measurements of hypochlorous acid production, using neutrophils or the myeloperoxidase/H_2O_2/Cl^- system, it was found that acetoacetate but not 3-hydroxybutyrate is able to inhibit the generation of this antimicrobial oxidant. The superoxide anion scavenging properties were confirmed by ferricytochrome C reduction and lucigenin-enhanced chemiluminescence assays. However, ketone bodies did not alter the rate of oxygen uptake by stimulated neutrophils, measured with an oxygen electrode. A strong inhibition of the expression of the cytokine IL-8 by cultured neutrophils was also observed; this is discussed with reference to the antioxidant-like property of acetoacetate.

Key words neutrophil; ketone body; hypochlorous acid; respiratory burst; myeloperoxidase; cytokine

Diabetes mellitus is a syndrome characterized by abnormal insulin secretion and derangement of carbohydrate and lipid metabolism; it is diagnosed by the presence of hyperglycemia.[1] In addition, type-1 diabetes patients frequently experience hyperketonemia caused by an increased fatty acid metabolism.[2] The blood concentration of ketone bodies may reach 10 mmol/l in patients with severe ketosis, compared with less than 0.5 mmol/l to normal individuals.[2,3] Diabetic patients with frequent episodes of ketosis also experience increased incidences of vascular diseases and the correlation of these with oxidative stress is widely accepted.[2—4] In this regard, it was demonstrated recently the involvement of ketone bodies as new factor involved in oxidative stress since acetoacetate (AcAc) can promote lipid peroxidation in human endothelial cells *via* the generation of oxygen radicals and also lower the level of glutathione and increase peroxidation in human erythrocytes.[5,6] These deleterious properties of ketone bodies and particularly of AcAc are readily correlated with their ability to be oxidized. This chemical property prompts interaction with reactive oxygen species produced at sites of inflammation and, as a consequence, promotes their suppression or the generation of new deleterious compound. In fact, abnormally high levels of AcAc inhibit luminol-enhanced neutrophil chemiluminescence when these cells are stimulated, and this may have a direct effect in the microbial function of these cells.[7] On the other hand, the generation of a carbon-centered radical was demonstrated when myeloperoxidase-catalyzed peroxidation of AcAc proceeds in the presence of the piperidinooxy free radical, which could lead to a pro-oxidant activity.[8]

The role of the neutrophil enzyme myeloperoxidase (MPO) as a promoter of oxidative damage, *via* the generation of hypochlorous acid (HOCl) is well known. This powerful oxidant agent reacts with unsaturated fatty acids or cholesterol generating chlorohydrins, leading to increased cell-membrane permeability. It promotes the oxidation of proteins through the chlorination of tyrosine and tryptophan residues leading to protein dysfunction. It also mediates the production of carbonyls groups on proteins *via* the breakdown of chloramines.[9—13] Hence, substances able to react with HOCl might inhibit these processes, but, on the other hand, they could lead to generation of new oxidant species with unknown deleterious properties. Ketone bodies with their oxidant-scavenging properties are liable to develop both of these effects, particularly in diabetic patients that suffer from ketosis during all their whole life.

In this paper we described the effect of ketone bodies on the production of reactive species and particularly hypochlorous acid. It is demonstrated that AcAc but not 3-hydroxubutyrate (3-HOB) is able to inhibit the production of HOCl by activated neutrophils and this effect is linked to direct competition with the MPO endogenous substrate chloride anion. In addition, the suppression of the pro-inflammatory cytokine IL-8 production when neutrophils were incubated with ketone bodies points to the still incompletely known effects of ketosis upon the inflammatory process.

MATERIALS AND METHODS

Chemicals Hydrogen peroxide 30% (w/w) was purchased from Peroxidos do Brazil (SP, Brazil). Myeloperoxidase (MPO) (E.C. 1.11.1.7), phorfol-12-myristate-13-acetate (PMA), superoxide dismutase (SOD) (E.C. 1.15.1.1), zymosan, Luminol, lucigenin, 3,3′,5,5′-tetramethylbenzidine (TMB), acetoacetate lithium salt, 3-hydroxybutyrate and Ficoll-Hypaque 1077 and 1113 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All the reagents used for buffer preparation were of analytical grade.

Solutions Hydrogen peroxide was prepared by diluting a 30% stock solution and its concentration was calculated from its absorption at 240 nm (ε=43.6 mol^{-1} l cm^{-1}).[14] Hypochlorous acid was prepared by diluting a concentrated commercial bleach solution and calculating its concentration from its absorption at 292 nm (ε=350 mol^{-1} l cm^{-1}).[15] The ketone bodies, luminol and lucigenin solutions were prepared by dissolving them in water. MPO stock solution was prepared by dissolving the lyophilized protein in water and its activity measured using the TMB substrate. PMA stock solution was prepared by dissolving it in DMSO. Opsonized zymosan was prepared as described.[16]

* To whom correspondence should be addressed. e-mail: fonseclm@fcfar.unesp.br

Isolation of Human Neutrophils The study was approved by the University of the State of São Paulo Ethics Committee and the healthy donors gave written informed consent. Neutrophils were isolated from blood of healthy donors by Ficoll-Hypaque centrifugation.[17] After isolation, neutrophils were resuspended in 10 mmol/l PBS-D (Dulbecco's buffer).

Chemiluminescent Assay for Cell System Neutrophils (1×10^6 cells/ml) were pre-incubated at 37 °C in PBS-D with 10 μmol/l luminol or 10 μmol/l lucigenin and various concentrations of AcAc or 3-HOB for 1 min. The reaction was started by adding 1 mg/ml opsonized zymosan. The chemiluminescence was measured for 30 min.

Chemiluminescent assay for enzyme system: MPO (100 mU/ml) was pre-incubated at 37 °C in PBS-D with 10 μmol/l luminol and various concentration of AcAc by 1 min. The reaction was started by adding 20 μmol/l H_2O_2. The chemiluminescence was measured for 5 min. The reactions were carried out in a BIOOrbit model 1251 luminometer (Turku, Finland).

Determination of Hypochlorous Acid Production by Neutrophils Neutrophils (2×10^6 cells/ml) were pre-incubated at 37 °C in PBS-D with 5 mmol/l taurine and various concentrations of AcAc for 10 min. Cells were stimulated by adding 100 ng/ml PMA. After 30 min the reaction was stopped by adding 20 μg/ml catalase. The neutrophils were pelleted by centrifugation and the supernatant put on ice. Formation of hypochlorous acid was measured by assaying accumulated taurine chloramine.

Determination of Hypochlorous Acid Production by MPO/H_2O_2/Cl^- The MPO (25 mU/ml) and various concentrations of AcAc were incubated in PBS-D with 5 mmol/l taurine. The reaction was started by addition 50 μM hydrogen peroxide. After 30 min the reaction was stopped by adding catalase (20 μg/ml). Formation of hypochlorous acid was measured by assaying accumulated taurine chloramine (see below).

Hypochlorous Acid Assay The accumulated taurine chloramine was assayed by adding 400 μl of neutrophil supernatant or 400 μl of MPO reaction system to 100 μl of a solution containing 10 mmol/l TMB dissolved in 50% dimethylformamide, 100 μmol/l potassium iodide and 400 mmol/l acetic acid. Under this condition taurine chloramine oxidizes TMB to a blue product with an absorbance maximum at 655 nm (Kettle *et al.* submitted). A standard curve was generated by adding pure HOCl to PBS-D containing taurine. The absorbance measurements were made in a HITACHI U-3000 spectrophotometer.

Measurements of Superoxide Production by Stimulated Neutrophils Superoxide was measured *via* SOD-inhibitable reduction of ferricytochrome C. Neutrophils (2×10^6 cells/ml) were pre-incubated for 10 min at 37 °C in PBS-D plus 20 μg/ml catalase and 100 μmol/l ferricytochrome C. Cells were stimulated by adding 100 ng/ml PMA and the change in absorbance at 550 nm was measured over the first minutes of the reaction.

Consumption of Oxygen by Activated Neutrophils Neutrophils (2×10^6 cells/ml) were incubated at 37 °C in PBS-D with or without 10 mmol/l AcAc. The oxygen monitor was set to 100% and the reaction started by adding 100 ng/ml PMA. The reactions were carried out in an oxygen monitor, Yellow Spring 5300 (YSI, U.S.A.).

Myeloperoxidase Activity The enzyme activity was assayed by adding MPO to 50 mmol/l phosphate buffer pH 5.4 with 1 mmol/l TMB (dissolved in dimethylformamide) and various AcAc concentrations at 25 °C. The reaction was started by adding 50 μmol/l H_2O_2. The change in absorbance at 650 nm was measure over the first minutes and the activity calculated as the rate of consumption of H_2O_2.

Cytokine Assay Neutrophils (2×10^6 cells/ml) suspended in Dulbecco's buffer were cultured at 37 °C and 5% CO_2 alone or with AcAc. After 24 h the supernatant was collected and the ELISA assay performed (Quantikine, R&D System, Minneapolis, MN, U.S.A.).[18]

Statistical Analysis Average values were expressed as the mean and standard deviation, and Student's *t*-test used to examine differences between the control and tests ($p<0.05$).

RESULTS

Luminol-enhanced chemiluminescence was used to assay the total reactive oxygen species (ROS) produced by stimulated neutrophil.[19] Typically the reactions had 1×10^6 cells/ml and they were triggered by opsonized zymosan. The chemiluminescence was measured for 30 min and the integrated signal taken as analytical parameter. The addition of either AcAc or 3-HOB caused the chemiluminescence to be suppressed in a concentration dependent manner. However, the effect was significantly stronger when AcAc was used (Fig. 1).

The luminol chemiluminescence that occurs when neutrophils are stimulated is not specific, since it may be the result of MPO catalyzed luminol oxidation or it could be generated by the reaction between luminol and HOCl produced in the medium.[20] Therefore, some assays were performed using purified MPO, hydrogen peroxide and chloride to find out the effect on the production of HOCl. The results in Fig. 2 show that AcAc is able to suppress the production of this oxidant specie, whereas with 3-HOB the effect was not significant. On the other hand, using this assay we can not specify if ketone bodies are reacting or inhibiting the generation of HOCl. Hence, to improve our knowledge on the role of AcAc in the inhibition of HOCl generate *via* MPO catalysis,

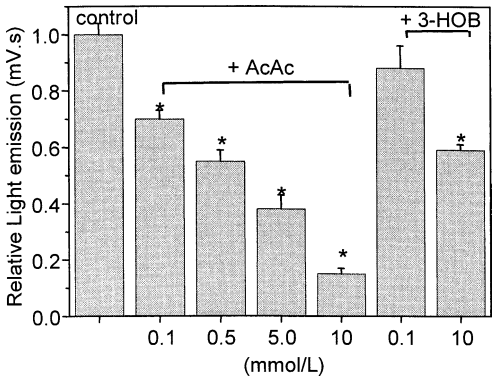

Fig. 1. The Effect of Ketone Bodies on Luminol-Enhanced Neutrophil Chemiluminescence

The data are relative to the control without ketone bodies. The reactions were started by adding zymosan (see Materials and Methods). The data represent the average±S.D. of 7 experiments using neutrophils from 3 healthy donors (*$p<0.05$ compared to the control).

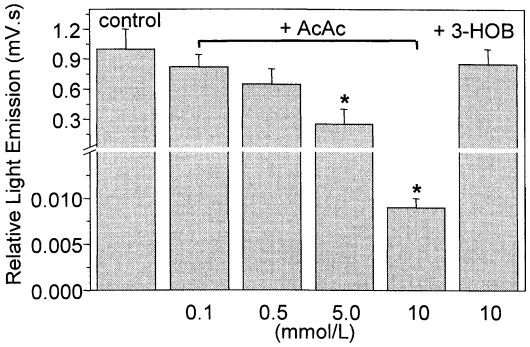

Fig. 2. The Effect of Ketone Bodies on Luminol-Enhanced MPO/Cl-Chemiluminescence

The data are relative to the control without ketone bodies. The reactions were started by adding H_2O_2 (see Materials and Methods). The data represent the average±S.D. of 6 experiments (*$p<0.05$ compared to the control).

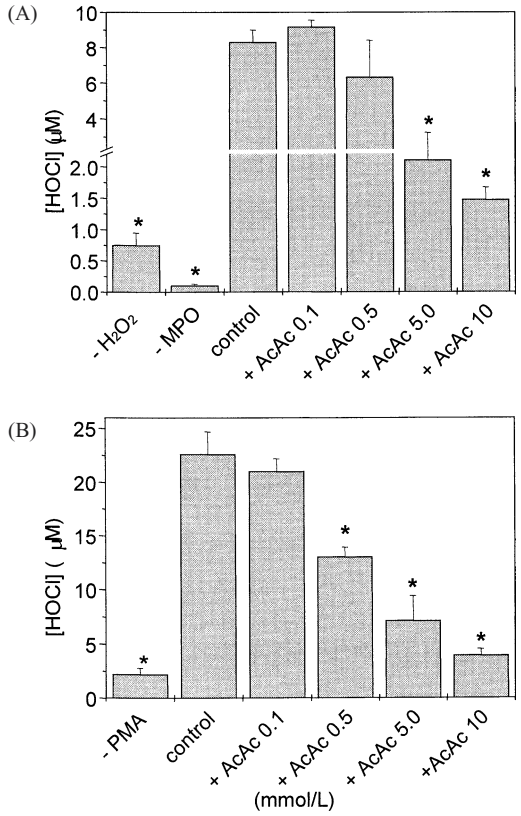

Fig. 3. The Effect of Acetoacetate on Hypochlorous Acid Production

(A) Effect on MPO/H_2O_2/Cl^- system. The data represent the average±S.D. of 3 experiments (*$p<0.05$ compared to the control). (B) Effect on activated neutrophil system. The data represent the average±S.D. of 7 experiments using neutrophils from 3 healthy donors (*$p<0.05$ compared to the control). See Materials and Methods for experimental conditions.

a quantitative and specific assay was performed by measuring the competition between AcAc and taurine for HOCl. Taurine reacts very fast with HOCl, so the competitive inhibition of this reaction gives a good idea of the reactivity of any other substrate with HOCl.[21] Firstly, pure HOCl (10 μmol/l) was added to the PBS buffer with taurine 5 mmol/l and AcAc 10 mmol/l. The formed taurine chloramine formed was revealed by the TMB assay (Kettle *et al.* submitted). The inhibition of the formation of taurine chloramine was only 13±2% (average of 3 experiments). The formed taurine chloramine did not react with AcAc (not shown).

Table 1. Inhibition of MPO Activity by Ketone Bodies

	MPO Activity (U/ml)
Complete system	4.7±0.4
+AcAc 0.1 mM	4.0±0.1
+AcAc 10 mM	2.6±0.1
+AcAc 20 mM	1.8±0.1
+3-HOB 10 mM	4.1±0.2

The data represent the average±S.D. of 3 experiments. For experimental conditions see Materials and Methods.

Table 2. The Effect of Acetoacetate on Superoxide Anion Production by Activated Neutrophils

	$[O_2^-]$ (μM/min)
Complete system	5.2±1.2
+AcAc 0.1 mM	4.4±0.9
+AcAc 10 mM	2.7±0.8

The data represent the average±S.D. of 7 experiments using neutrophils from 3 healthy donors. For experimental conditions see Materials and Methods.

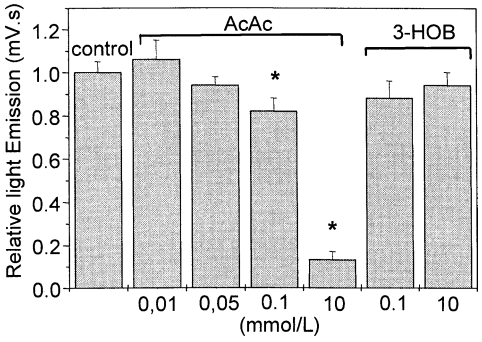

Fig. 4. The Effect of Ketone Bodies on Lucigenin-Enhanced Neutrophil Chemiluminescence

The data are relative to the control without ketone bodies. The reactions were started by adding zymosan (see Materials and Methods). The data represent the average±S.D. of 7 experiments using neutrophils from 3 healthy donors (*$p<0.05$ compared to the control).

Next, the MPO system was used to generate HOCl with taurine and AcAc present in the PBS buffer. The reaction was triggered by H_2O_2 and stopped after 30 min by the addition of catalase and revealed by TMB assay. The data in (Fig. 3A) show about 80% inhibition of HOCl when 10 mmol/l AcAc was added to the reaction medium. This is evidence that in these experimental conditions the direct reaction between AcAc and HOCl is not responsible for the total suppressive effect detected here. In a further step, neutrophils activated by PMA were used for HOCl generation and again a clear inhibition was detected (Fig. 3B). The inhibition of the MPO activity was additionally confirmed through measurement of the peroxidase activity. A concentration of 10 mmol/l AcAc caused an inhibition of about 50% (Table 1).

Ketone bodies have also been described as scavengers of superoxide anion.[21] To detect this effect, the lucigenin chemiluminescence assay was performed, as it is supposed to be linked to anion superoxide generated during the respiratory burst.[19,20] The results in (Fig. 4) show that AcAc in high concentration, but not 3-HOB, is a scavenger of superoxide anion in this experimental model. The spectrophotometric ferricytochrome C assay confirmed the lucigenin experiment

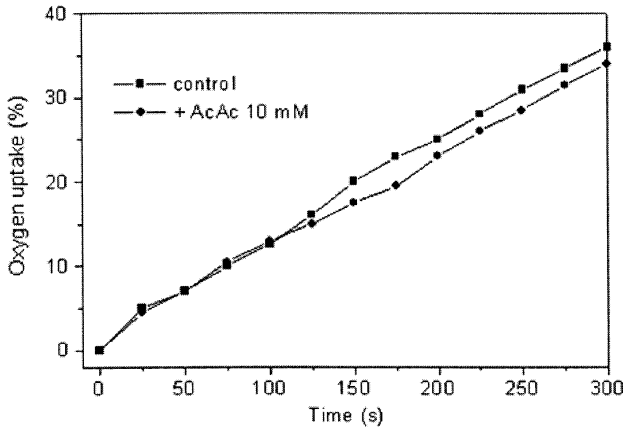

Fig. 5. Oxygen Uptake by Activated Neutrophils
The traces are representative of 2 experiments (see Materials and Methos).

Table 3. The Effect of Acetoacetate on IL-8 Production by Neutrophils

	IL-8 (pg/ml)
Complete system	3800±100
+AcAc 0.1 mM	3400±200
+AcAc 10 mM	1550±30

The data represent the average±S.D. of 3 experiments. For experimental conditions see Materials and Methods.

(Table 2). The effect of AcAc is not related to the generation of superoxide anion, since the oxygen uptake when neutrophils are stimulated by PMA was not affected by addition of AcAc (Fig. 5).

The antioxidant-like effect of AcAc upon the reactive species generated by stimulated neutrophils leads to consider whether it could have an effect on the expression of inflammatory mediators by these cells. The expression of the cytokine IL-8 by cultured neutrophils stimulated by PMA was measured to test this hypothesis. A clear suppressive effect can be observed in Table 3.

DISCUSSION

It is well established that ketone bodies suppress the generation of reactive species when neutrophils are exposed to stimuli like opsonized particles, microorganisms or the soluble protein kinase C activator, PMA. Usually these studies involve luminol chemiluminescence techniques to measure the total and non-specific generation of reactive species. The observed decrease in the light signal when AcAc or 3-HOB are added to the reaction medium is explained by the scavenging of oxidant species by these metabolites.[7,22] An inspection of the chemical structures of AcAc and 3-HOB leads to the conclusion that the latter should be less susceptible to oxidation, since the target group in this molecule (alcohol) is not usually oxidized by reactive species generated during the respiratory burst and neither are alcohol groups substrates of peroxidases. In this work we confirmed this hypothesis, finding that 3-HOB is a weaker scavenger than AcAc. On the other hand, as would be expected of a 1,3-dicarbonyl compound whose oxidizable enol tautomer is in equilibrium with the keto form,[23]AcAc shows an unquestionable reactive species suppressor property.

In our opinion the remaining question is: what reactive species generated in the phagocyte is really reacting with AcAc? Using a quantitative and more specific assay to measure the production of HOCl, we have approached this question. The specificity of the taurine/TMB assay is based on the ability of HOCl reacts with taurine forming taurine chloramine. The high concentration of taurine (5 mmol/l) and the high rate constant to the reaction between this compound and HOCl ($(4.8\pm0.1)\times10^5\,mol^{-1}\,l\,s^{-1}$) ensure that taurine will be the main target for the HOCl formed in the enzymatic or cell system when using this assay.[24] In these experimental conditions the addition of a test substrate can affect the production of taurine chloramine in three different ways: i) competing with the taurine for HOCl; ii) reacting with taurine chloramine; iii) competing with the chloride anion by compound-I of MPO. Although our controls showed that AcAc is really able to react directly with pure HOCl, this does not seem to be the only reaction, since AcAc can not compete efficiently with taurine. Thus, our results point to real competition between the chloride anion and AcAc for the catalytic site of MPO, which, as a consequence, inhibits the formation of HOCl. To verify if AcAc could be a good substrate to MPO, it was studied its inhibitory properties using a classical substrate of peroxidases, the TMB assay. The rate constants of reaction between AcAc and the active forms of MPO known as compound-I and II have not been determined yet. However, considering the values of k_1 ($(3.6\pm0.1)\times10^6\,mol^{-1}\,l\,s^{-1}$) and k_2 ($(9.4\pm0.6)\times10^5\,mol^{-1}\,l\,s^{-1}$) for the reaction between TMB and compounds I and II of MPO respectively,[25] the inhibition obtained in the presence of AcAc is evidence that it can be a good substrate for this enzyme. The antioxidant property of AcAc and particularly its inhibiting of HOCl production could have a strong influence on the antimicrobial activity of the neutrophil. Indeed, a mechanism was recently proposed for virulence of *Candida albicans* based upon its ability to produce high levels of pyruvate, potentially resulting in localized tissue ketosis and undermining the normal defensive function of neutrophil MPO.[26]

Here we have confirmed previous results that pointed to a scavenging action by AcAc on superoxide anions generated by neutrophil.[22] More recently, the demonstration of the auto-oxidation of AcAc, leading to the production of superoxide, raised the number of reactions known to be mediated by this ketone body which may have consequences for the redox balance of hyperketonemia-suffering subjects.[5,6] Hence, our own and previous results indicate that AcAc is able both to scavenge and to generate superoxide. This is not completely surprising, considering the low reduction potential of superoxide anion which enables it to be easily oxidized and reduced. Moreover, our results indicated that AcAc cannot inhibit the multi-enzyme complex NADPH-oxidase and consequently does not affect the production of superoxide anion, but only reacts with it.

Although we did not perform an ESR experiment, our results are in agreement with previous ESR results,[8] since the efficiency with which AcAc competes with the chloride anion is also a strong indication that a reactive carbon-centered radical of AcAc can really be expected in this experimental model and is also a possible reactive species formed *in vivo* by type-1 diabetic patients suffering from inflammatory diseases. Besides this, the posterior products of radical

reaction such as an intermediate hydroperoxide or the final product methylglyoxal could have deleterious effects on the inflammatory site by promoting lipid peroxidation and protein dysfunction. Indeed, AcAc has been shown to promote of lipid peroxidation in erythrocytes and increase the lipid peroxidation in human endothelial cells *via* a superoxide anion mediated reaction.[5,6] Moreover, using a model of inflammation (peritonitis) with MPO knockout mice, it was demonstrated that this enzyme could be the responsible for initiation of lipid peroxidation at sites of inflammation.[27]

Endogenous oxidizing species such as H_2O_2 and HOCl play an important role in the pathophysiology of inflammatory disease, not only as terminal effectors, but also as secondary messengers in signal transduction. HOCl induced tumor necrosis factor-alpha production in peripheral blood mononuclear cells in a tyrosine kinase-dependent manner.[28] H_2O_2 increases expression of mRNA of various chemokines and macrophage-inflammatory proteins.[29] In this connection, antioxidants have been tested as suppressors or promoters of cytokine expression in cell systems. For example, *N*-acetylcysteine, decreased the TNF-α induced expression of intercellular adhesion molecule-1 on cultured epithelial cells from human bronchi, and inhibited IL-8 production by those cells.[30] This may represent a parallel to the action of AcAc since it was an inhibitor of liberation of IL-8 by neutrophil. This inhibition is one more indication of the harmful role played by ketone bodies with direct consequences in diabetic patients.

In conclusion, our results suggest an efficient mechanism by which radical species and/or organic peroxide could be formed during an inflammatory condition, which are really frequent in diabetic patients. Considering also the generation of superoxide anion *via* the endothelial NADPH-oxidase system and the presence of MPO in the endothelium, secreted by activated neutrophils, the oxidation of AcAc gains importance and may mediate the diabetes vascular complications more than we currently suppose.

Acknowledgments This study was supported by Laboratório de Análises Clínicas, UNESP, Araraquara (LAC) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), the Brazilian Federal Co-ordinating Council for Highest Education.

REFERENCES

1) Zimmet P. Z., McCarty D. L., Courten M. P., *J. Diabetes Complicat.*, **11**, 60—68 (1997).
2) Vignati L., Asmal A. C., Black W. L., Brink S. J., Hare J. W., "Diabetes Mellitus," eds. by Kahn C. R., Weir G. C., Lea & Febiger, Philadelphia, 1985, pp. 526—552.
3) Candiloros H., Muller S., Zeghari N., Donner M., Drouin P., Ziegler O., *Diabetes Care*, **18**, 549—551 (1995).
4) White N. H., *Endocrinol. Metab. Clin. North Am.*, **29**, 657—682 (2000).
5) Jain S. K., Kannan K., Lim G., *Free Radic. Biol. Med.*, **25**, 1083—1088 (1998).
6) Jain S. K., McVie R., *Diabetes*, **48**, 1850—1855 (1999).
7) Saeed F. A., Castle G. E., *Clin. Diagn. Lab. Immunol.*, **5**, 740—743 (1998).
8) Harrison J. E., Saeed F. A., *Biochem. Med.*, **29**, 149—163 (1983).
9) Heinecke J. W., Li W., Mueller D. M., Bohrer A., Turk J., *Biochemistry*, **23**, 10127—10136 (1994).
10) Spickett C. M., Jerlich A., Panasenko O. M., Arnhold J., Pitt A. R., Stelmaszynska T., Schaur R. J., *Acta Biochim. Pol.*, **47**, 889—899 (2000).
11) Domigan M. N., Charlton T. S., Duncan M. W., Winterbourn C. C., Kettle A. J., *J. Biol. Chem.*, **270**, 16542—16548 (1995).
12) Carr A. C., Decker E. A., Park Y., Frei B., *Free Radic. Biol. Med.*, **31**, 62—72 (2001).
13) Chapman A. L., Winterbourn C. C., Brennan S. O., Jordan T. W., Kettle A. J., *Biochem. J.*, **375**, 33—40 (2003).
14) Desser R. K., *Arch. Biochem. Biophys.*, **148**, 452—465 (1972).
15) Brestel E. P., *Biochem. Biophys. Res. Commun.*, **126**, 482—488 (1985).
16) Allen L. A., Yang C., Pessin J. E., *J. Leukoc. Biol.*, **72**, 217—221 (2002).
17) English D., Andersen B. R., *J. Immunol. Methods*, **5**, 249—255 (1974).
18) Furlaneto C. J., Ribeiro F. P., Hatanaka E., Souza G. M., Cassatella M. A., Campa A., *Lipids*, **37**, 925—928 (2002).
19) Dahlgren C., Karlsson A., *J. Immunol. Methods*, **232**, 3—14 (1999).
20) Allen R. C., Dale D. C., Taylor F. B., Jr., *Methods Enzymol.*, **305**, 591—629 (2000).
21) Kettle A. J., Winterbourn C. C., *Methods Enzymol.*, **233**, 502—512 (1994).
22) Sato N., Shimizu H., Shimomura Y., Suwa K., Mori M., Kobayashi I., *Life Sci.*, **51**, 113—118 (1992).
23) Adam W., Baader W. J., Cilento G., *Biochim. Biophys. Acta*, **88**, 330—336 (1986).
24) Folkes L. K., Candeias L. P., Wardman P., *Arch. Biochem. Biophys.*, **323**, 120—126 (1995).
25) Marquez L. A., Dunford H. B., *Biochemistry*, **36**, 9349—9355 (1997).
26) Saeed F. A., *FEMS Microbiol. Lett.*, **190**, 35—38 (2000).
27) Zhang R., Brennan M. L., Shen Z., MacPherson J. C., Schmitt D., Molenda C. E., Hazen S. L., *J. Biol. Chem.*, **277**, 46116—46122 (2002).
28) Schieven G. L., de Fex H., Stephenson L., *Antioxid. Redox Signal*, **4**, 501—507 (2002).
29) Jaramillo M., Olivier M., *J. Immunol.*, **169**, 7026—7038 (2002).
30) Ishii Y., Yamasawa H., Kitamura S., *Nihon Kyobu Shikkan Gakkai Zasshi*, **34**, 126—130 (1996).