British Journal of Nutrition (2009), **101**, 1351–1359
doi:10.1017/S0007114508076307

Modulation of rat neutrophil function *in vitro* by *cis*- and *trans*-MUFA

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(Received 2 April 2008 – Revised 20 August 2008 – Accepted 21 August 2008 – First published online 2 October 2008)

In the present study, the effects of *trans*-MUFA, elaidic acid (EA; 18 : 1-9*t*) and vaccenic acid (VA; 18 : 1-11*t*) on rat neutrophil functions were compared with those of *cis*-monounsaturated oleic acid (OA) (18 : 1-9*c*) and saturated stearic acid (SA; 18 : 0) (10–150 μM). *Trans*-fatty acids enhanced neutrophil phagocytic capacity, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide production, and candidacidal activity. The same effects were observed for OA. Cells treated with *trans*-MUFA showed reduced production of $NO^{\cdot}$, whereas those treated with OA showed an increase in production. Treatment with SA did not provoke significant effect on the parameters investigated. The increase in $O_2^{\cdot-}$ production induced by MUFA was not observed when diphenyleneiodonium, an NADPH oxidase inhibitor, was added to the medium. This finding suggests that MUFA stimulate neutrophil NADPH oxidase activity. The addition of 3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione, a protein kinase C (PKC) inhibitor, and wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor, did not affect $O_2^{\cdot-}$ production induced by MUFA. Therefore, the mechanisms by which MUFA stimulate NADPH oxidase are not dependent on PKC and do not seem to involve PI3K. Experiments using Zn^{2+}, an inhibitor of NADPH oxidase H^+ channel, indicated that MUFA activate the NADPH oxidase complex in rat neutrophil due to opening of H^+ channel.

Neutrophils: *Trans*-MUFA: Superoxide anion: NADPH oxidase: Phagocytosis

In the diets of developed nations, one-quarter of total daily energies is provided by fatty acids that contain one or more double bonds in the molecules. These double bonds are typically positioned in the 3, 6, 7 or 9 carbon atoms from the terminal methyl group. Their geometry is usually *cis*, i.e. the two hydrogen atoms on the carbons adjacent to the double bond are located in the same side of the carbon chain, resulting in a bent shape and a liquid state at room temperature. However, some fatty acids have one or more double bonds in the *trans* configuration, i.e. the hydrogen atoms on the carbons adjacent to the double bonds are in opposite sides, resulting in a straight configuration and a solid state at room temperature. *Trans*-fatty acids are abundant in dairy fat and ruminant meats, but the main source is partially hydrogenated vegetable or fish oils. Production of partially hydrogenated fats to form margarines and shortenings began early in the twentieth century and increased steadily until about the 1960s. The processed vegetable fats displaced animal fats in the diets of most people in industrialised countries. The initial motivation was lower cost, but health benefits were also purported. The average per person consumption of *trans*-fatty acids from partially hydrogenated oils has remained at about 2 % of energies since the 1960s, because of the increased use of these fats in commercially baked products and fast foods. Since 1990, there has been increasing public-health concern about epidemiological studies, showing that *trans*-fatty acids increase the risk of CHD such as acute myocardial infarction, cardiovascular risk factors and sudden cardiac death[1–3].

Neutrophils are the first cells that migrate to tissues in response to invading micro-organisms. The antimicrobial function of these phagocytes depends on the release of lytic enzymes stored in cytoplasmatic granules and on the production of reactive oxygen species (ROS). In phagocytes, superoxide is mainly generated by the reaction of oxygen and NADPH through the NADPH oxidase complex[4–6]. This enzyme system comprises of cytosolic components, $p47^{phox}$ and $p67^{phox}$, a low molecular weight G-protein, Rac2 and a membrane-associated cytochrome *b*558. In resting neutrophils, the subunits of the oxidase complex are distributed between cytosol (granules) and the membranes. When the phagocytes are activated, cytosolic components become heavily phosphorylated and migrate to the membrane, where they bind to cytochrome *b*558 to assemble the active oxidase. Superoxide anion and hydrogen peroxide (H_2O_2) generated by the NADPH oxidase complex give rise to strong cytolytic agents, such as hypochlorous acid and hydroxyl

Abbreviations: DPI, diphenyleneiodonium; EA, oleic acid; GF109203X, 3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; NBT, nitroblue tetrazolium; OA, elaidic acid; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PMA, phorbol myristic acetate; ROS, reactive oxygen species; SA, stearic acid; VA, vaccenic acid; ZY, zymosan.

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radical. Neutrophil activation involves a variety of plasma membrane receptors that interact with several compounds including bacterial products, components of the complement and soluble compounds such as cytokines released by other cells. The cascade that leads to ROS production by neutrophils involves G-protein and via phospholipase C the release of inositol trisphosphate and diacylglycerol. Diacylglycerol activates protein kinase C (PKC) that catalyses the phosphorylation of $p47^{phox}$. NADPH oxidase can also be activated via PI3K. There is evidence that PI3K can regulate the assembly and activity of the neutrophil NADPH oxidase complex *in vitro*, acting to recruit the enzyme components to phagosome[7].

Fatty acids have been shown to regulate immune and inflammatory responses[8–10]. In fact, fatty acids have been employed for the treatment of various oxidative stresses involving diseases, such as CHD and rheumatoid arthritis[11,12]. A recent study from our laboratory has shown that the C18 fatty acids (OA, linoleic and γ-linolenic acids) stimulate ROS production, through NADPH oxidase activation, by human and rat neutrophils[6]. Evidence has been accumulated that OA and linoleic acid either *in vivo* and *ex vivo* induce leucocyte death[13,14], and this effect may occur through oxidative stress[15–17]. Therefore, these fatty acids are able to modulate neutrophil function[18,19] in addition to their well-known effects on macrophage function, lymphocyte proliferation, cytokine production and modification of natural killer cell activity[20–23].

The afore-mentioned information led us to investigate the effect of C18 *trans*-MUFA (VA and EA) on rat neutrophil function. The results were compared with those of C18 OA (*cis*-monounsaturated) and SA (saturated). The following neutrophil functions were studied: phagocytic capacity; production of ROS; candidacidal activity. The involvement of NADPH oxidase, PKC, phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase and NADPH oxidase H^+ channel in the effects of the fatty acids was also examined.

Materials and methods

Chemicals

Fatty acids were purchased from MP Biochemicals LLC, Irvine, CA; wortmannin, 3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (GF109203X), diphenyleneiodonium (DPI), rotenone, nitroblue tetrazolium (NBT), zymosan (ZY) A (*Saccharomyces cerevisiae*), oyster glycogen type II, cytochrome *c* and sodium nitrite were obtained from Sigma-Aldrich Corporation, St Louis, MO, USA; Roswell Park Memorial Institute (RPMI)-1640 medium and fetal calf serum were from Invitrogen, Carlsbad, CA, USA.

Animals

Male Wistar rats weighing 180–200 g were used throughout the study. These animals were obtained from the Institute of Biomedical Sciences, University of São Paulo. The procedure used in the present study was approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo.

Peritoneal cell preparation

Male Wistar rats weighing 180–200 g were killed by decapitation without anaesthesia. Neutrophils were obtained by intraperitoneal lavage with 40 ml sterile PBS, 4 h after the intraperitoneal injection of 20 ml sterile oyster glycogen solution (1 %) in PBS. The cells were centrifuged (850 ***g*** for 10 min) three times in PBS. The number of viable neutrophils, over 95 % of the total cells harvested from intraperitoneal cavity, was counted in a Neubauer chamber by optical microscopy, using a 1 % Trypan blue solution in saline[24–26].

Culture of neutrophils

The cells ($5{\cdot}0 \times 10^6$ cells/ml) were seeded in RPMI-1640 medium supplemented with 10 % (v/v) fetal calf serum, 2 mM-glutamine and 20 μg penicillin–streptomycin per ml. The medium was supplemented with OA, EA, VA and SA dissolved in ethanol (0·5 %) at final concentrations of 10–150 μM, as indicated in the Results section. This range of fatty acid concentration was chosen so as to highlight the differences in the effects of C18 fatty acid on neutrophil function. The fatty acids were fully soluble in the concentrations and conditions used.

*Preparation of zymosan (*Saccharomyces cerevisiae*) for the measurement of phagocytosis*

Thirty-five milligrams of ZY in 100 ml PBS were boiled for 30 min and washed twice with PBS before use. For opsonisation, 0·5 ml ZY particles (14 mg/ml PBS) were mixed with 0·5 ml rat serum and incubated for 30 min at 37°C. The opsonised ZY particles were then washed and resuspended in PBS at a concentration of 1 mg/ml.

Phagocytosis assay

Neutrophils, which were previously cultured with fatty acids, were incubated (2×10^6 cells per flask) in 1 ml PBS with 2 % (w/v) defatted bovine serum albumin, in the presence of glucose (5 mM), containing opsonised ZY at 37°C for 30 min. The percentage of phagocytosis was determined by counting the cells that had phagocytosed three or more particles of ZY in a counting chamber. We used a procedure similar to that used in our previous studies[24,27].

Neutrophil candidacidal activity assay

The candidacidal activity was assessed by the method described by Lehrer & Cline[28]. Neutrophils (1×10^6 cells per flask) were incubated in 1 ml RPMI-1640 medium with 10 % (v/v) fetal calf serum, containing 75 μM OA, EA, VA or SA in the presence of *Candida albicans* (1:1 proportion) for 60 min at 37°C. A control sample was treated with 0·5 % ethanol instead of the fatty acids. After incubation, 100 μl Triton X-100 (5·5 %) were added followed by the addition of 1·9 ml methylene blue solution (0·01 %). At this concentration, Triton causes immediate lysis of neutrophils without causing damage to the *Candida* cells, whose viability was assessed by methylene blue exclusion in a Neubauer chamber. The candidacidal activity was expressed as a percentage of stained yeast cells in relation to total. Similar procedure was used in our previous study[27].

Measurement of hydrogen peroxide production

The measurement of H_2O_2 production was performed in the cells that were previously incubated with 100 μM fatty acids.

H_2O_2 production was measured by using the method of phenol red as described by Pick & Mizel[29]. This is based on the horseradish peroxidase-dependent conversion of phenol red by H_2O_2 into a coloured compound. Briefly, the cells were incubated in the presence of phenol red and horseradish peroxidase, under an atmosphere of 5 % CO_2–95 % air at 37°C during 1 h. After this period, the reaction was stopped by adding 10 μl NaOH solution (1 M) and the amount of H_2O_2 formed was measured at 620 nm (Spectramax plus; Molecular Devices, Sunnyvale, CA, USA). The production of H_2O_2 was always determined against standard curves prepared for each experiment.

Nitroblue tetrazolium reduction

Superoxide production was estimated by reduction of NBT, a yellow water-soluble powder that becomes blue and insoluble upon reduction[30,31]. Cells (4×10^6 cells/ml) were incubated for 1 h at 37°C in 0·1 % NBT solution in phosphate-buffered saline with glucose solution: 0·13 mM-NaCl, 2·7 mM-KCl, 1·0 mM-$MgCl_2$, 5 mM-glucose and 10 mM-NaH_2PO_4/Na_2HPO_4, pH 7·4. The reaction was stopped by placing the samples on ice. After cell centrifugation, reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication (one pulse of 5 s). Cell debris was pelleted and absorbance of the supernatant was determined at 560 nm in a microtitre plate reader (Spectramax plus; Molecular Devices). Freshly obtained neutrophils were incubated for the determination of NBT reduction in the presence of phorbol myristic acetate (PMA; 162 nM) and GF109203X (5 μM). A similar procedure was used in our previous study[15].

The main advantage of this method is the possibility to determine both intra- and extracellular superoxide production. Reducing agents such as NO^- can also reduce NBT and could jeopardise the measurement of $O_2^{-\cdot}$ production[32]. In a control experiment, the cells were pretreated with *N*-nitro-L-Arg, an inhibitor of NO synthase. The addition of *N*-nitro-L-Arg to the incubation medium did not affect the results of NBT reduction (data not shown).

Protein determination

Protein content of cell preparations was measured by the method of Bradford[33], using bovine serum albumin as a standard.

Statistical analysis

All the experiments were performed in triplicate and repeated at least three times. The results are presented as means and standard deviations. Statistical significance of the differences between groups was assessed by the two-tailed, unpaired Student's *t* test or ANOVA as appropriate. Significance was considered for $P<0·05$.

Results

Phagocytosis capacity

Neutrophils treated with 100 μM EA and VA had their phagocytic capacity enhanced by 51 %, whereas OA caused an increase of 61 % (Table 1). The addition of SA to the incubation medium did not cause any effect.

Candidacidal activity

Trans-MUFA increased the killing of opsonised *C. albicans* by neutrophils by 30–40 %, after 60 min incubation, when compared with control cells treated with 0·5 % ethanol (Table 2). Stearic acid also did not cause a significant effect.

Hydrogen peroxide production

MUFA increased H_2O_2 production by PMA-stimulated neutrophils but had no effect on unstimulated cells (Fig. 1). Stearic acid did not increase H_2O_2 production when compared with the control group.

Determination of nitroblue tetrazolium reduction

Neutrophils exposed to 100 μM MUFA for 1 h showed a 3-fold increase in NBT reduction when compared with the control. Stearic acid also promoted an increase in NBT reduction but at much less extend by 60 % (Fig. 2). To better characterise the effect of the fatty acids on superoxide production, dose–response curves and kinetics of NBT reduction were performed (Figs. 3 and 4, respectively). The concentrations used were 10, 20, 50, 100 and 150 μM and the incubation periods were 15, 30, 45 and 60 min. As shown by the dose–response curve, the effect of the fatty acids on NBT reduction was as follows: OA > EA = VA > SA (Fig. 4). The effect of the fatty acids was observed already after 15 min incubation (about 70 % increase), and it did not markedly change afterwards (Fig. 5).

Mechanisms by which oleic, elaidic and vaccenic acids enhance superoxide anion production

In order to investigate whether superoxide production induced by the fatty acids involves the activation of this system, an NADPH oxidase inhibitor, DPI, was used[34]. The cells were also treated with two compounds that activate NADPH oxidase by different mechanisms, PMA and ZY. PMA

Table 1. Effect of the fatty acids on neutrophil phagocytic capacity†
(Mean values and standard deviations of nine determinations)

Treatment	Percentage of phagocytosis	
	Mean	SD
Control (0·5 % ethanol)	46·4	5·4
OA	74·8*	6·7
EA	71·4*	3·7
VA	71·4*	3·7
SA	52·9	5·6

OA, oleic acid; EA, elaidic acid; VA, vacceric acid; SA, stearic acid; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline.
*Mean value was significantly different from that of the control ($p < 0·05$).
† Neutrophils (5×10^6 cells/ml) were incubated for 3 h at 37°C in RPMI-1640 culture medium in the presence of the fatty acids at 100 μM or 0·5 % ethanol. After this period, the cells were pelleted and washed twice with PBS. The cells (2×10^6 cells/ml) were then incubated for 40 min in PBS containing 5 mM-glucose, 2 % albumin and zymosan particles.

Table 2. Effect of the fatty acids on neutrophil candidacidal activity†
(Mean values and standard deviations of nine determinations)

Treatment	Percentage of dead *Candida* cells	
	Mean	SD
Control (0·5 % ethanol)	23·0	3·0
OA	30·2*	2·2
EA	31·2*	2·8
VA	32·4*	4·4
SA	28·5	2·6

OA, oleic acid; EA, elaidic acid; VA, vacceric acid; SA, stearic acid; RPMI, Roswell Park Memorial Institute.
*Mean value was significantly different from that of the control ($p < 0·05$).
†Neutrophils (1×10^6 cells/ml) were incubated for 3 h at 37°C in 1 ml RPMI-1640 culture medium in the presence of the fatty acids at 100 μM or 0·5 % ethanol (control) and *Candida albicans* at 1:1 proportion. After this period, 100 μl Triton-X-100 (5·5 %) and 1·9 ml methylene blue (0·01 %) were added to the incubation medium to evaluate the proportion of dead *Candida* cells.

activates PKC, which phosphorylates $p47^{phox}$, a cytosolic component of NADPH oxidase, initiating the activation of the NADPH oxidase complex[35]. ZY, in turn, interacts with a membrane receptor and leads to NADPH oxidase activation.

The decrease in NBT reduction observed in the cells treated with PMA and ZY stimulation was much greater than that induced by the treatment with MUFA. DPI caused a 70 % reduction in superoxide production induced by the fatty acids (Fig. 6). This effect of DPI was still more pronounced in the presence of PMA and ZY (Fig. 6).

Neutrophils were also treated with GF109203X, a PKC inhibitor[36]. PMA-induced NBT reduction was completely abolished by GF109203X, whereas the effect of ZY was only partially reversed (Fig. 7). However, GF109203X did not affect the NBT reduction induced by the fatty acids.

In order to investigate whether MUFA induce NBT reduction through PI3K activation, neutrophils were pre-incubated

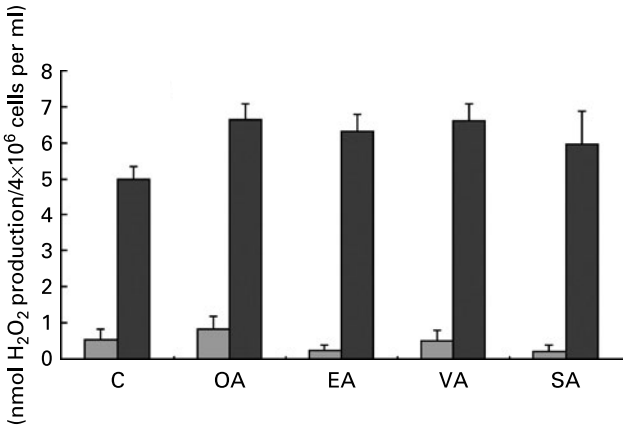

Fig. 1. Production of hydrogen peroxide (H_2O_2) by neutrophils treated with OA, EA, VA or SA. Cells (4×10^6 cells/ml) were incubated for 3 h in Roswell Park Memorial Institute (RPMI)-1640 culture medium with 10 % fetal calf serum and 100 μM fatty acids. Control cells were incubated with 0·5 % ethanol. The bars represent the results of H_2O_2 production obtained under basal (□) and phorbol myristic acetate-stimulated (■) conditions. The values are presented as means and standard deviations of nine determinations (n 9). *Mean value was significantly different from that of the control ($p < 0·05$). C, control; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid; SA, stearic acid.

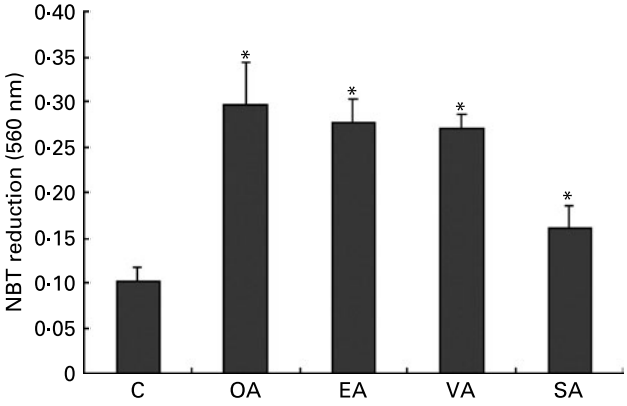

Fig. 2. Nitroblue tetrazolium (NBT) reduction by neutrophils incubated in the presence of OA, EA, VA or SA. Neutrophils (4×10^6 cells/ml) were incubated for 1 h at 37°C in phosphate-buffered saline with glucose solution containing 0·1 % NBT in the presence of 100 μM fatty acids under constant stirring. The reaction was stopped by placing the samples in ice. After centrifugation, reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication (one pulse of 5 s). Cell debris was pelleted and absorbance of the supernatant was determined at 560 nm. The values are expressed as means and standard deviations of nine determinations (n 9). *Mean value was significantly different from that of the control ($p < 0·05$). C, control; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid; SA, stearic acid.

with wortmannin, an inhibitor of PI3K[37]. The NBT reduction induced by PMA was significantly reduced and that by ZY was fully abolished. However, fatty acid-induced NBT reduction was not affected by the treatment with wortmannin (Fig. 7).

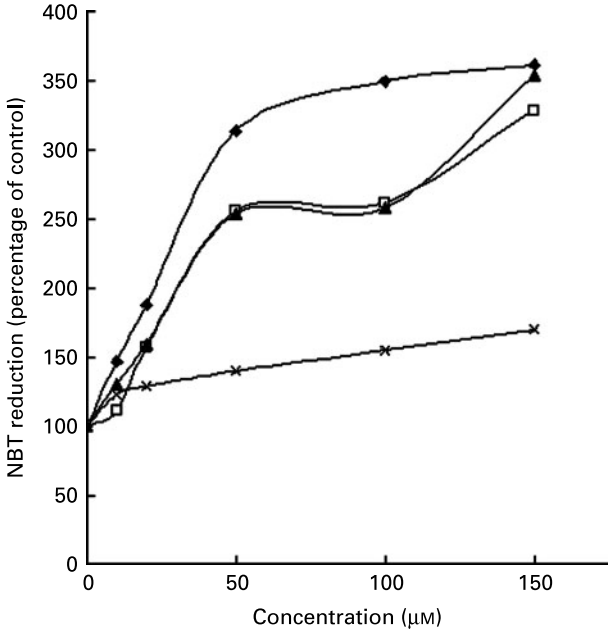

Fig. 3. Dose–response curve of nitroblue tetrazolium (NBT) reduction in neutrophils incubated in the presence of oleic (–◆–), elaidic (–□–), vaccenic (–▲–) or stearic (– × –) acid. Neutrophils (4×10^6 cells/ml) were treated with different concentrations (10, 20, 50, 100 and 150 μM) of fatty acids in phosphate-buffered saline with glucose solution containing 0·1 % NBT for 1 h at 37°C under constant stirring. After centrifugation, reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication. Cell debris was pelleted and absorbance of the supernatant was determined at 560 nm. The results are expressed as percentage of increase when compared with the control group (100 %) from two experiments.

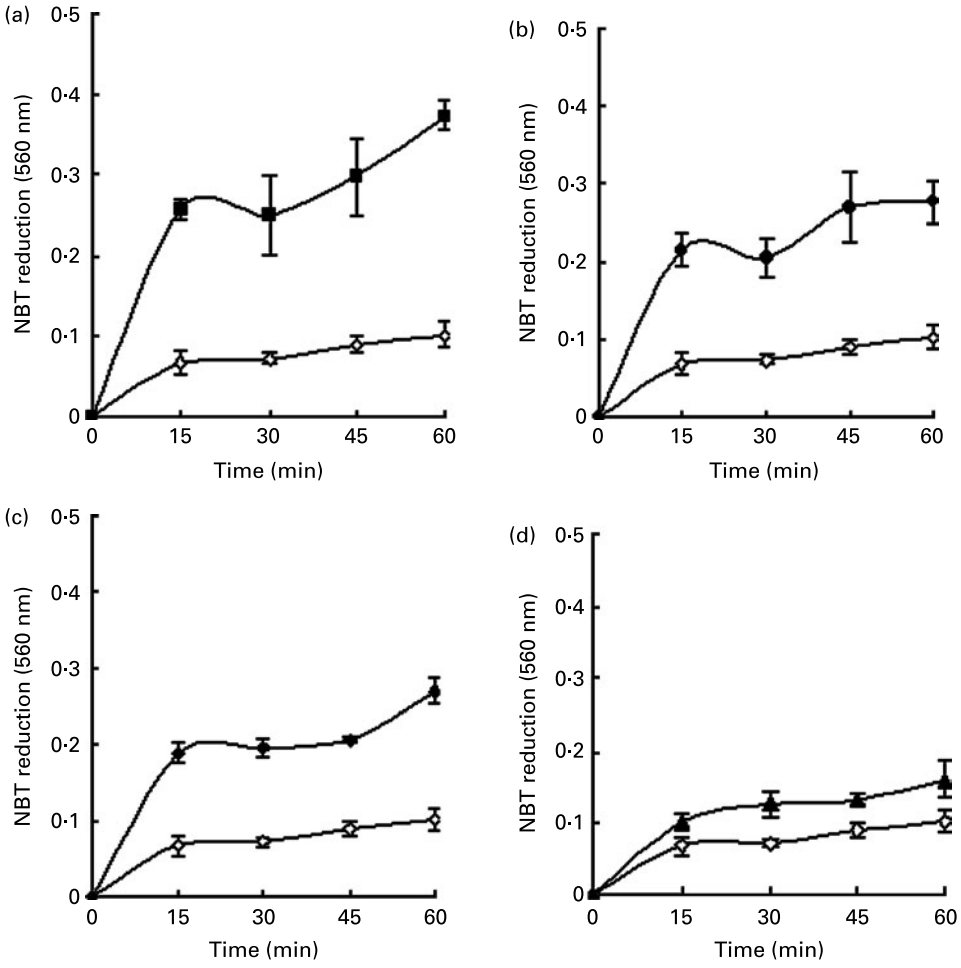

Fig. 4. Kinetics of nitroblue tetrazolium (NBT) reduction induced by (a) oleic, –■–; ethanol, –◇–, (b) elaidic, –◆–; ethanol, –◇–, (c) vaccenic, –◆–; ethanol, –◇– and (d) stearic, –▲–; ethanol, –◇– acids in incubated neutrophils. Neutrophils (4×10^6 cells/ml) were incubated in the presence of the fatty acids (75 μM) in phosphate-buffered saline with glucose solution containing 0·1% NBT for 15, 30, 45 and 60 min at 37°C under constant stirring. After centrifugation, reduced NBT was solubilised in 100 μl acetic acid solution (50%) upon sonication. Cell debris was pelleted and absorbance of the supernatant was determined at 560 nm. The results are expressed as means and standard deviations of nine determinations (*n* 9).

The administration of Zn^{2+}, an H^+ channel inhibitor[38,39], caused a 60% decrease in the fatty acid- and ZY-induced NBT reduction, whereas the decrease in the reduction induced by PMA was only 30% (Fig. 8).

The priming role of the MUFA on the PMA and ZY responses needs to be better discussed with respect to previous reports on this aspect.

Discussion

The effect of C18 *cis*- and *trans*-monounsaturated and SFA on neutrophil function was investigated. Phagocytic capacity of neutrophils was increased by MUFA, and this effect did not depend on the double-bond space configuration *cis* or *trans*. The position of the double bond in the aliphatic chain did not have any influence either. In fact, neutrophil phagocytosis capacity was not different between EA (18 : 1-9*t*) and VA (18 : 1-11*t*).

Neutrophils treated with *cis*- and *trans*-MUFA for 3 h showed a significant increase of H_2O_2 production. Thus, the increase in phagocytic capacity induced by MUFA was also followed by an increment of H_2O_2 production. Again, SA did not cause important change in H_2O_2 production. So far, the presence of double bonds in the C18 fatty acid regardless of the *cis* or *trans* configuration seems to play an important role in neutrophil activation.

Treatment with *cis*- and *trans*-MUFA also enhanced candidacidal activity. This result indicates that the increase in phagocytic capacity and ROS production effectively contributes to micro-organism destruction. Other fatty acids have also been shown to control neutrophil cytolytic activity. Neutrophils treated with long-chain PUFA, in particular arachidonic, eicosapentaenoic and DHA, have their antiparasitic activity against *Plasmodium falciparum* enhanced[40]. On the other hand, bacterial killing activity of neutrophils is significantly reduced by the treatment with medium-chain triacylglycerol emulsion[23,41,42].

Neutrophils pre-exposed to the MUFA showed an increased response to PMA (Fig. 1), suggesting that these fatty acids prime the cells. Similar findings have been previously reported for PUFA[43,44]. This priming effect may be associated with the increased neutrophil phagocytosis capacity and microbial killing activity induced by MUFA treatment.

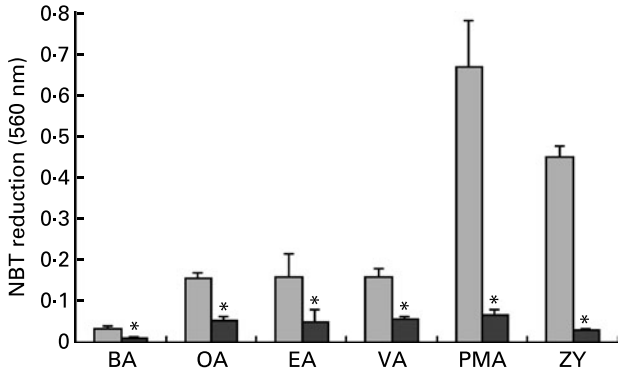

Fig. 5. Effect of diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, on nitroblue tetrazolium (NBT) reduction in neutrophils treated with fatty acids, phorbol myristic acetate (PMA) or zymosan (ZY). Cells (4×10^6 cells/ml) were pre-incubated in phosphate-buffered saline with glucose (PBSG) solution with 0·5 % ethanol (control;□) or DPI chloride (20 μM; ■) for 30 min at 37°C. One volume of PBSG solution containing 0·1 % NBT, 0·5 % ethanol, fatty acids (150 μM), PMA (40 nM) or ZY (100 particles per cell) was added and the cells were then incubated at 37°C for 1 h. After this period, the cells were pelleted and reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication. After centrifugation, absorbance of the supernatant was determined at 560 nm. The results are expressed as means and standard deviations of nine determinations (*n* 9). BA, basal; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid; SA, stearic acid; PMA, phorbol myristic acetate; ZY, zymosan. *$P<0·05$ due to the effect of DPI.

Hydrogen peroxide is formed upon dismutation of superoxide anion ($O_2^{-\cdot}$). The exposure of neutrophils to MUFA caused a marked increase of NBT reduction. This difference can be explained by the fact that the basal values of $O_2^{-\cdot}$ production were about four times greater in pre-incubated neutrophils when compared with freshly obtained ones. Therefore, cells pre-incubated with MUFA show increased capacity to produce $O_2^{-\cdot}$. Oleic acid had a slightly more pronounced effect than *trans*-fatty acids on NBT reduction. Previous study has shown that OA stimulates neutrophil adherence and degranulation even at lower concentrations[45].

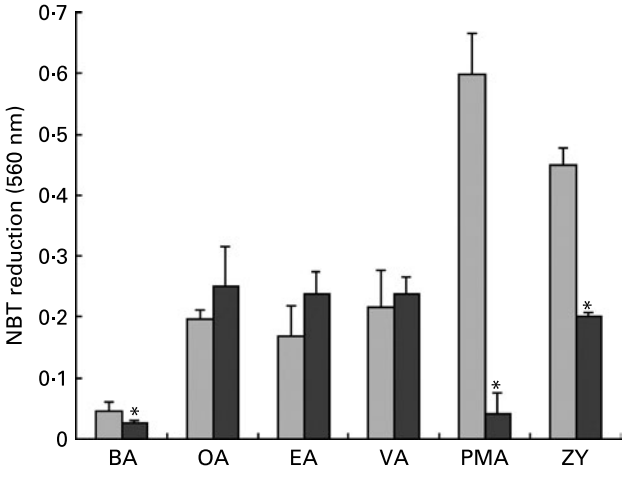

Fig. 6. Effect of 3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (GF109203X), a protein kinase C inhibitor, on nitroblue tetrazolium (NBT) reduction induced by the fatty acids, phorbol myristic acetate (PMA) and zymosan (ZY). Neutrophils (4×10^6 cells/ml) were incubated in phosphate-buffered saline with glucose (PBSG) solution containing 0·5 % DMSO (control;□) or GF109203X (10 μM; ■) for 30 min at 37°C. One volume of PBSG solution containing 0·1 % NBT, 0·5 % DMSO, fatty acids (150 μM), PMA (40 nM) or ZY (100 particles per cell) was added and the cells were then incubated at 37°C for 1 h. After this period, the cells were pelleted and reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication. After centrifugation, absorbance of the supernatant was determined at 560 nm. The results are expressed as means and standard deviations of nine determinations (*n* 9). *$P<0·05$ due to the effect of GF109203X. BA, basal; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid; SA, stearic acid; PMA, phorbol myristic acetate; ZY, zymosan.

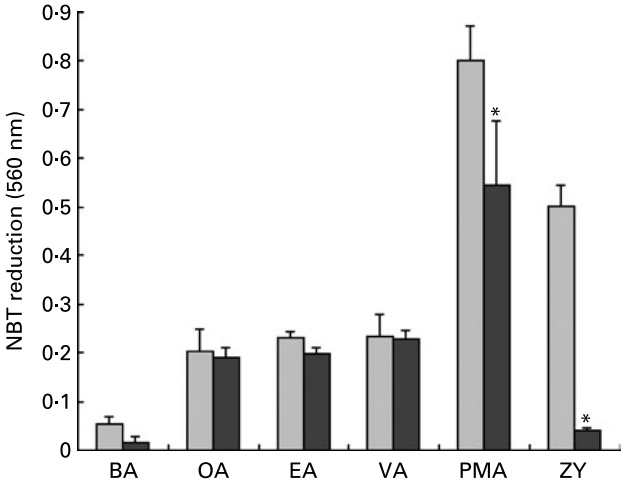

Fig. 7. Effect of wortmannin, a phosphatidylinositol-3 kinase inhibitor, on nitroblue tetrazolium (NBT) reduction induced by fatty acids, phorbol myristic acetate (PMA) or zymosan (ZY). Neutrophils (4×10^6 cells/ml) were incubated in phosphate-buffered saline with glucose (PBSG) solution in the absence (PBS;□) or presence of wortmannin (200 nM; ■) for 30 min at 37°C. One volume of PBSG solution containing 0·1 % NBT, fatty acids (150 μM), PMA (40 nM) or ZY (100 particles per cell) was added and the cells were again incubated at 37°C for 1 h. After this period, the cells were pelleted and reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication. After centrifugation, absorbance of the supernatant was determined at 560 nm. The results are expressed as means and standard deviations of nine determinations (*n* 9). *$P<0·05$ due to the effect of wortmannin. BA, basal; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid; PMA, phorbol myristic acetate; ZY, zymosan.

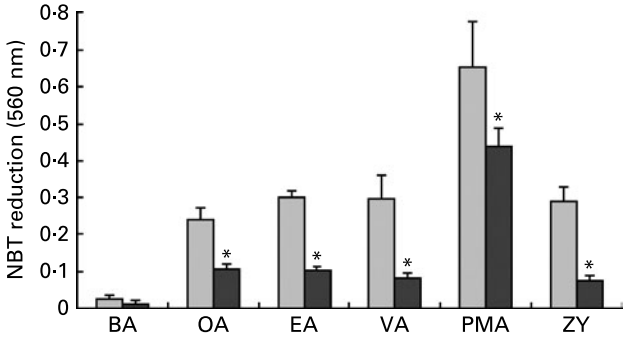

Fig. 8. Effect of zinc, an NADPH oxidase H^+ channel inhibitor, on nitroblue tetrazolium (NBT) reduction induced by fatty acids, phorbol myristic acetate (PMA) or zymosan (ZY)-stimulated neutrophils. Neutrophils (4×10^6 cells/ml) were incubated in phosphate-buffered saline with glucose (PBSG) solution in the absence (phosphate-buffered saline; □) or in the presence of (zinc chloride 100 μM; ■) for 30 min at 37°C. One volume of PBSG solution containing 0·1 % NBT, fatty acids (150 μM), PMA (40 nM) or ZY (100 particles per cell) was added and the cells were again incubated at 37°C for 1 h. After this period, the cells were pelleted and reduced NBT was solubilised in 100 μl acetic acid solution (50 %) upon sonication. After centrifugation, absorbance of the supernatant was determined at 560 nm. The results are expressed as means and standard deviations of nine determinations (*n* 9). *$P < 0·05$ due to the effect of Zn^{2+}. BA, basal; OA, oleic acid; EA, elaidic acid; VA, vaccenic acid.

Badway *et al.*[46] reported that *cis*-unsaturated fatty acids stimulate the release of $O_2^{-\cdot}$ from neutrophils. This effect was not observed for saturated SA. Other studies[47–49] showed that the intensity of the response increases with the unsaturation degree of the fatty acids. The present observations and those from others support the proposition that superoxide production induced by C18 fatty acids in neutrophils increased with the presence of unsaturation in the molecule, but was not influenced by the position of the double bond in the carbon chain. By contrast, however, at lower concentrations (from 2·5 to 10 μM), *cis*- and *trans*-MUFA had opposite effects on neutrophil function[50]. The differences in the rate of incorporation of the fatty acids into the plasma membrane under these latter conditions may explain these observations.

Diphenyleneiodonium, an NADPH oxidase inhibitor, lowered NBT reduction induced by MUFA. The same effect of DPI was observed in the cells treated with PMA and ZY, confirming that the source of superoxide was NADPH oxidase. Although DPI can inhibit several other flavoproteins, such as those of the mitochondrial electron transport chain, a specific inhibitor of this superoxide-producing site (rotenone) was ineffective to abolish the increase in NBT reduction induced by the fatty acids (data not shown). These results suggest that *cis*- and *trans*-MUFA stimulate ROS production through the activation of NADPH oxidase in neutrophils.

Nitroblue tetrazolium reduction induced by PMA was strongly inhibited also by the GF109203X treatment. This drug also inhibited NBT reduction in cells stimulated with ZY. However, NBT reduction induced by OA, EA and VA was not altered by GF109203X. These observations support the proposition that activation of NADPH oxidase by MUFA did not involve PKC as one could expect[51,52]. *Trans*- and *cis*-MUFA also did not activate respiratory burst in neutrophils through the activation of PI3K. Wortmannin treatment completely inhibited NBT reduction induced by ZY. However, the drug did not affect NBT reduction in cells treated with OA, EA and VA. These findings indicate that PI3K is not the mechanism involved in the activation of neutrophil NADPH oxidase induced by *trans*- and *cis*-MUFA. This contrasts with the requirement for PKC[53] and PI3K[54] with PUFA such as arachidonic acid.

The possibility that MUFA may lead to opening of NADPH oxidase H^+ channel in neutrophils was investigated. Single-electron transfer from internal NADPH to external oxygen is an electrogenic process and the efflux of H^+ through the H^+ channel is necessary for charge compensation[38]. The channel is voltage gated, i.e. the depolarisation is required to initiate H^+ efflux. However, the H^+ channel may be gated by ΔpH or by a combination of both membrane potential and ΔpH, i.e. the proton motive force. Henderson & Chappell[55] reported that arachidonate activates the H^+ channel. The addition of Zn^{2+}, an H^+ channel inhibitor, caused a 60 % decrease in superoxide production stimulated by the fatty acids. These findings indicate that fatty acids may activate NADPH oxidase via the H^+ channel. It remains to be investigated whether MUFA activate NADPH oxidase directly or by releasing arachidonic acid via phospholipase A_2 stimulation.

The results presented herein indicate that OA, EA and VA stimulate neutrophil phagocytosis, candidacidal activity and production of ROS. The effect of the fatty acids on ROS production occurred by the activation of NADPH oxidase through the H^+ channel opening. There was no marked difference in the effects of *cis*- and *trans*-MUFA, but SA had no significant effect. These findings support the proposition that undesirable effects of *trans*-MUFA, such as induction of insulin resistance[56], may be associated with their pro-inflammatory actions.

Acknowledgements

The authors declare no conflict of interest. R. P. developed the experimental work of his PhD thesis and R. C. was his supervisor. The present research has been supported by FAPESP, CNPq, CAPES and UNICSUL University. The authors are grateful to Drs L. R. Lopes, S. C. Sampaio and C. M. Peres for their valuable contribution during the development of the experiments and to the technical assistance of J. R. Mendonça and G. de Souza. The authors are indebted to E. P. Portioli Silva for revision and final preparation of the manuscript.

References

1. Steinhert H, Rickert R & Winkler K (2003) *Trans* fatty acids (TFA): occurrence, intake and clinical relevance. *Eur J Med Res* **8**, 358–362.
2. Roberts TL, Wood DA, Riemersma RA, *et al.* (1995) *Trans* isomers of oleic and linoleic acids in adipose tissue and sudden cardiac death. *Lancet* **345**, 278–282.
3. Merchant AT, Kelemen LE, de Koning L, *et al.* (2008) Share and Share AP investigators Interrelation of saturated fat, *trans* fat, alcohol intake, and subclinical atherosclerosis. *Am J Clin Nutr* **87**, 168–174.
4. Babior BM (1999) NADPH oxidase: an update. *Blood* **93**, 1464–1476.
5. Lopes LR, Laurindo FRM, Mancini-Filho J, *et al.* (1999) NADPH oxidase activity and lipid peroxidation in neutrophils from rats fed fat-rich diets. *Cell Biochem Funct* **17**, 57–64.
6. Hatanaka E, Levada-Pires AC, Pithon-Curi TC, *et al.* (2006) Systematic study on ROS production induced by oleic, linoleic, and γ-linolenic acids in human and rat neutrophils. *Free Radic Biol Med* **41**, 1124–1132.
7. Marshall JG, Booth JW, Stambolic V, *et al.* (2001) Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during FC gamma receptor-mediated phagocytosis. *J Cell Biol* **153**, 1369–1380.
8. Yaqoob P & Calder PC (2007) Fatty acids and immune function: new insights into mechanisms. *Br J Nutr* **98**, S41–S45.
9. Martins de Lima T, Gorjão R, Hatanaka E, *et al.* (2007) Mechanisms by which fatty acids regulate leucocyte function. *Clin Sci (Lond)* **113**, 65–77.
10. Gorjão R, Cury-Boaventura MF, de Lima TM, *et al.* (2007) Regulation of human lynphocyte proliferation by fatty acids. *Cell Biochem Funct* **25**, 305–315.
11. Wanten GJA & Calder PC (2007) Immune modulation by parenteral lipid emulsions. *Am J Clin Nutr* **85**, 1171–1184.
12. Calder PC (2006) *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 1505S–1519S.
13. Cury-Boaventura MF, Kanunfre CC, Gorjão R, *et al.* (2006) Mechanisms involved in Jurkat cell death induced by oleic and linoleic acids. *Clin Nutr* **25**, 1004–1014.
14. Cury-Boaventura MF, Gorjão R, de Lima TM, *et al.* (2008) Effect of olive oil-based emulsion on human lymphocyte and neutrophil death. *JPEN J Parenter Enteral Nutr* **32**, 81–87.

15. Pompéia C, Cury-Boaventura MF & Curi R (2003) Arachidonic acid triggers an oxidative burst in leukocytes. *Br J Med Biol Res* **36**, 1549–1560.
16. Azevedo-Martins AK & Curi R (2008) Fatty acids decrease catalase activity in human leukaemia cell lines. *Cell Biochem Funct* **26**, 87–94.
17. Patel C, Ghanim H, Ravishankar S, *et al.* (2007) Prolonged reactive oxygen species generation and nuclear factor-kappa B activation after a high-fat, high-carohydrate meal in the obese. *J Clin Endocrinol Metab* **92**, 4476–4479.
18. Pereira LM, Hatanaka E, Martins EF, *et al.* (2007) Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats. *Cell Biochem Funct* **26**, 197–204.
19. Kang JH, Lee GS, Jeung EB, *et al.* (2007) *Trans*-10, *cis*-12-conjugated linoleic acid increases phagocytosis of porcine peripheral blood polymorphonuclear cells *in vitro*. *Br J Nutr* **97**, 117–125.
20. Otton R, Graziola F, Souza JA, *et al.* (1998) Effect of dietary fat on lymphocyte proliferation and metabolism. *Cell Biochem Funct* **16**, 253–259.
21. Gorjao R, Hirabara SM, de Lima TM, *et al.* (2007) Regulation of IL-2 signaling by fatty acids in human lymphocytes. *J Lipid Res* **48**, 2009–2019.
22. Miles EA, Banerjee T, Dooper MM, *et al.* (2004) The influence of different combinations of gamma-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *Br J Nutr* **91**, 893–903.
23. Waitzberg CL, Lotierzo PH, Logullo AF, *et al.* (2002) Parenteral lipid emusions and phagocytic systems. *Br J Nutr* **87**, S49–S57.
24. Pithon-Curi TC, Melo MP, Palanch AC, *et al.* (1998) Percentage of phagocytosis, production of O_2, H_2O_2 and NO, and antioxidant enzyme activities of rat neutrophils in culture. *Cell Biochem Funct* **16**, 43–49.
25. Lagranha CJ, Hirabara SM, Curi R, *et al.* (2007) Glutamine supplementation prevents exercise-induced neutrophil apoptosis and reduces p38 MAPK and JNK phosphorylation and p53 and caspase 3 expression. *Cell Biochem Funct* **25**, 563–569.
26. Alba-Loureiro TC, Hirabara SM, Mendonça JR, *et al.* (2006) Diabetes causes marked changes in function and metabolism of rat neutrophils. *J Endocrinol* **188**, 295–303.
27. Sampaio SC, Sousa-e-Silva MCC, Borelli P, *et al.* (2001) Crotalus durissus terrificus snake venom regulates macrophage metabolism and function. *J Leuk Biol* **70**, 551–558.
28. Lehrer R & Cline MJ (1969) Interaction of *Candida albicans* with human leukocytes and serum. *J Bacteriol* **98**, 996–1004.
29. Pick E & Mizel D (1981) Rapid microasays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* **46**, 211–226.
30. Madhavi N, Das UN, Prabha PS, *et al.* (1994) Supression of human T-cell growth *in vitro* by *cis*-unsaturated fatty acids: relationship to free radicals and lipid peroxidation. *Prostaglandins Leukot Essent Fatty Acid* **51**, 33–40.
31. Schrenzel J, Serrander L, Banf B, *et al.* (1998) Electron currents generated by the human phagocyte NADPH oxidase. *Nature* **392**, 734–737.
32. Vasquez-Vivar J, Hogg N, Martasek P, *et al.* (1999) Tetrahydrobiopterin-depedent inhibition of superoxide generation from neuronal nitric oxide synthase. *J Biol Chem* **274**, 26736–26742.
33. Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
34. O'Donnell VB, Tew DG & England PJ (1993) Studies on the inhibitory mechanismo of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* **290**, 41–49.
35. El Benna J, Han J, Park J, *et al.* (1996) Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component p47phox by p38 and ERK but not JNK. *Arch Biochem Biophys* **334**, 395–400.
36. Toullec D, Pianetti P, Coste H, *et al.* (1991) The bisindolylmaleimide GF 109293X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**, 15771–15781.
37. Ellson C, Davidson K, Anderson K, *et al.* (2006) PtdIns3P binding to the PX domain of p40phox is a physiological signal in NADPH oxidase activation. *EMBO J* **25**, 4468–4478.
38. Henderson LM (1998) Role of histidines identified by mutagenesis in the NADPH oxidase-associated H^+ channel. *J Biol Chem* **273**, 33216–33223.
39. Levy R, Lowenthal A & Dana R (2000) Cytosolic phospholipase A_2 is required for the activation of the NADPH oxidase associated H^+ channel in phagocyte-like cells. *Adv Exp Med Biol* **479**, 125–135.
40. Kumaratilake LM, Ferrante A, Robinson BS, *et al.* (1997) Enhancement of neutrophil-mediated killing of *Plasmodium falciparum* asexual blood forms by fatty acids: importance of fatty acid structure. *Infect Immunol* **65**, 4152–4157.
41. Bellinati-Pires R, Waitzberg DL, Salgado MM, *et al.* (1993) Functional alterations of human neutrophils by medium-chain triglyceride emulsions: evaluation of phagocytosis, bacterial killing, and oxidative activity. *Lab Immunol* **53**, 404–410.
42. Wanten GJ, Curfs JH, Meis JF, *et al.* (2001) Phagocytosis and killing of *Candida albicans* by human neutrophils after exposure to structurally different lipi emulsion. *JPEN J Parent Enteral Nutr* **25**, 9–13.
43. Ferrante A, Carman K, Nandoskar M, *et al.* (1996) Cord blood neutrophil responses to polyunsaturated fatty acids: effects on degranulation and oxidative burst. *Biol Neonate* **69**, 368–375.
44. Huang ZH, Hii CS, Rathjen DA, *et al.* (1997) *n*-6 and *n*-3 Polyunsaturated fatty acids stimulate translocation of protein kinase C alpha, -beta, -betaII and epsilon and enhance agonist-induced NADPH oxidase in macrophages. *Biochem J* **325**, 553–557.
45. Bates EJ, Ferrante A, Smithers L, *et al.* (1995) Effect of fatty acid structure on neutrophil adhesion, degranulation and damage to endothelial cells. *Atherosclerosis* **116**, 247–259.
46. Badway JA, Curnute JT, Robinson JM, *et al.* (1984) Effects of fre fatty acids on release of superoxide and on change of shape by human neutrophils: reversibility by albumin. *J Biol Chem* **259**, 7870–7877.
47. Poulos A, Robinson BS, Ferrante A, *et al.* (1991) Effect of 22–32 carbon *n*-3 polyunsaturated fatty acids on superoxide production in human neutrophils: synergism of docosahexaenoic acid with f-met–leu–phe and phorbol ester. *Immunology* **73**, 102–108.
48. Hardy SJ, Ferrante A, Poulos A, *et al.* (1994) Effect of exogenous fatty acids with greater than 22 carbon atoms (very long chain fatty acids) on superoxide production by human neutrophils. *J Immunol* **153**, 1754–1761.
49. Robinson BS, Hll CST & Ferrante A (1998) Activation of phospholipase A2 in human neutrophils by polyunsaturated fatty acids and its role in stimulation of superoxide production. *Biochem J* **336**, 611–617.
50. Steinbeck MJ, Robinson JM & Karnovsky MJ (1991) Activation of the neutrophil NADPH-oxidase by free fatty acids requires the ionized carboxyl group and partitioning into membrane lipid. *J Leukoc Biol* **49**, 360–368.
51. Bosca L, Diaz-Guerra MJ & Mojena M (1989) Oleate-induced translocation o protein kinase C to hepatic microsomal membranes. *Biochem Biophys Res Commun* **160**, 1243–1249.
52. Padma M & Das UN (1999) Effect of *cis*-unsaturated fatty acids on the activity of protein kinases and protein phosphorylation in macrophage tumor (AK-5) cells *in vitro*. *Prostaglandins Leukot Essent Fatty Acid* **60**, 55–63.

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53. Hii CS, Huang ZH, Bilney A, *et al.* (1999) Involvement of protein kinase C, p38 MAP Kinase and ERK in arachidonic acid-stimulated superoxide production in human neutrophils. *Adv Exp Med Biol* **469**, 365–370.
54. Hii CST, Moghadammi N, Dunbar A, *et al.* (2001) Activation of the phosphatidylinositol 3-kinase-Akt/protein kinase B signaling pathway in arachidonic acid-stimulated human myeloid and endothelial cells. *J Biol Chem* **276**, 27246–27255.
55. Henderson LM & Chappell JB (1992) The NADPH-oxidase-associated H+ channel is opened by arachidonate. *Biochem J* **283**, 171–175.
56. Simopoulos AP (1994) Is insulin influenced by dietary linoleic acid and *trans* fatty acids? *Free Radic Biol Med* **17**, 367–372.

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