Vol. 152, No. 1, 1988
April 15, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 301-307

EFFECT OF SUPEROXIDE AND LIPID PEROXIDE ON CYTOSOLIC FREE CALCIUM CONCENTRATION IN CULTURED PIG AORTIC ENDOTHELIAL CELLS

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Received March 2, 1988

Summary: The effect of reactive oxygen on cytosolic free calcium concentration ($[Ca^{++}]i$) in pig aortic endothelial cells (ECs) was studied. Linoleate hydroperoxide (LHO) and superoxide radicals generated from xanthine with xanthine oxidase (X-XO) were used as sources of reactive oxygen. $[Ca^{++}]i$ in ECs was measured with quin 2 and the value for quiescent ECs was 112 ± 11 nM. Both LHO and X-XO increased $[Ca^{++}]i$ in a dose-dependent manner without accompanying the significant cellular damage. Nifedipine suppressed the increase in $[Ca^{++}]i$ provoked by LHO and X-XO. Thus, the biological effects of reactive oxygen might be mediated, at least in part, by the activation of voltage-dependent calcium channels in ECs.

The importance of vascular endothelial cells (ECs) in preventing the development of atherosclerosis has been well documented. The damage of ECs is considered as a possible promotor of atherosclerotic process (1). Reactive oxygen spieces are known to be a risk factor contributing to atherogenesis (2,3). The cellular mechanism, however, has not been fully understood, although reactive oxygen has been reported to affect the calcium metabolism in ECs and to stimulate the transport of macromolecules such as albumin (4). Thus, in order to test the hypothesis that cytosolic calcium is involved in the action of reactive oxygen to ECs, we studied the effect of superoxide and linoleate hydroperoxide on $[Ca^{++}]i$ in ECs.

Abbreviations: $[Ca^{++}]i$: cytosolic free calcium concentration, BSA: bovine serum albumin, FCS: fetal calf serum, HBSS: Hank's balanced salt solution, $[^3H]$2-DOG: $[^3H]$2-deoxy-D-glucose, GSH: glutathione, GPox: glutathione peroxidase, LHO: linoleate hydroperoxide, MEM: minimal essential medium, SOD: superoxide dismutase, X: xanthine, XO: xanthine oxidase

0006-291X/88 $1.50

MATERIALS AND METHODS

Chemicals

SOD, catalase, xanthine, xanthine oxidase, GSH, GPox and soybean lipoxidase were obtained from Sigma (USA), [^{3}H]2-DOG from New England Nuclear (USA), quin 2/AM from Wako Chemicals (Japan), and linoleate from Serdary Research Laboratories (Canada). Nifedipine was provided by Bayer (FRG).

Cell Culture

ECs were obtained from the thoracic aorta of six-month-old male pig by scraping the luminal surface with a razor blade (5). Cells were cultured in MEM supplemented with 10% FCS at 37°C in 5% CO_2 and 95% air. Cells at 3 to 7 passages were used for the experiment. Cells were identified as being ECs from microscopic observation and from the incorporation of DiI-acetylated low density lipoprotein (6).

Preparation of Linoleate Hydroperoxide

LHO was prepared according to the procedure described by Thomas and Pryor (7). Briefly, 0.22 g of linoleate was dissolved in 0.7 ml of ethanol and further dissolved in 100 ml of borate buffer (0.05 M, pH 9). Soybean lipoxidase (4050 units/ml) was added and the mixture was incubated for 10 minutes at 0°C. The reaction was terminated by adding HCl. LHO was then extracted with 20% ether-hexane 3 times, evaporated at 24°C, and dissolved in 1 ml of ethanol. LHO was subsequently stored at -20°C until the time of the experiment. The LHO activity was assayed employing the method described by Yagi (8).

Estimation of Endothelial Cell Injury

Endothelial cell injury was estimated based on the release of incorporated [^{3}H]2-DOG from the ECs (9). Briefly, ECs were inoculated onto 24 multi-well plastic dishes (Inter Med, Nunc) and were cultured until confluency was reached. [^{3}H]2-DOG (1 μCi/ml) was added to the culture medium and incubated for 18 hours. Cells were washed with HBSS containing 0.5 % BSA. LHO or X-XO dissolved in HBSS containing 0.5% BSA was incubated along with the ECs at 37°C for 1 hour. Radioactivities in the medium and in the cell lysate obtained by treatment with 0.2 ml of Triton X-100 (2%) were measured with a liquid scintillation counter (Aloka LSC-700, Japan). The specific release of [^{3}H]2-DOG from the ECs was defined as 100x(A-C)/(B-C) (%), where A is the radioactivity in the medium of treated cells, B is the total radioactivity in the medium and cell lysate of the treated cells, and C is the radioactivity in the control cell medium.

Measurement of Cytosolic Free Calcium Concentration

[Ca^{++}]i in ECs was measured according to the method reported by Tsien et al. (10) and Capponi et al. (11) with minor modification as described elsewhere (12). The fluorescence of quin 2-loaded cells was measured by Hitachi 650-10S fluorescence spectrophotometer. LHO or X-XO was added , quickly mixed and fluorescence was measured once or twice per minute for 10 minutes. ECs were pretreated respectively with nifedipine ($3.2x10^{-6}$M) or scavenger enzymes 10 minutes and 1 minute before the addition of LHO or X-XO. At the end of fluorescence measurement, 20 μM of digitonin was added to the cells to measure the Fmax, or the fluorescence intensity of calcium-saturated quin 2 (13). Fmin, or the fluorescence intensity of calcium-free quin 2 was calculated from the equation; Fmin = 0.16 x (Fmax - background) + background. The background, that is, the autofluorescence of cells and coverslips, was finally measured by adding 0.5 mM of $MnCl_2$. The [Ca^{++}]i was calculated from the equation; [Ca^{++}]i = Kd x (F - Fmin)/(Fmax - F). Kd, previously determined by Tsien et al., was 115 nM (10).

Statistics

Data were analyzed with one-factor analysis of variance. When the statistically significant effects were found, Newman-Keuls tests were

performed to isolate differences between groups. A p value of less than 0.05 was considered to be significant. All data are presented in the text and figures as mean ± SEM.

RESULTS

Cytosolic Free Calcium Concentration

$[Ca^{++}]i$ in quiescent ECs was found to be 112 ± 11 nM (n=30). Both X (0.1 mM)-XO (2.5-40 mU/ml) and LHO (0.032-1 μM) increased $[Ca^{++}]i$ in a dose-dependent manner and peak values were obtained at 5-7 minutes after the addition of X-XO or LHO. Linoleate, even at 100 μM, did not affect $[Ca^{++}]i$ in ECs. As shown in Fig. 1, both SOD and SOD-catalase significantly suppressed the increase in $[Ca^{++}]i$ provoked by X-XO. Nifedipine ($3.2x10^{-6}$M) also suppressd it. The effect of GSH-GPox was not significant. As shown in Fig. 2, nifedipine and GSH-GPox suppressed the increase in $[Ca^{++}]i$ provoked by LHO (0.32 μM). The effect of SOD-catalase was not significant.

Endothelial Cell Injury

As shown in Fig. 3, the specific release of $[^3H]$2-DOG caused by X-XO at various concentrations was less than 10%, and the values were no higher than that produced by 0.1 mM X alone. As shown in Fig. 4, the specific release by LHO in doses of 0.032 to 0.32 μM also remained less than 10%, although 1 and 10 μM LHO produced significantly higher specific release.

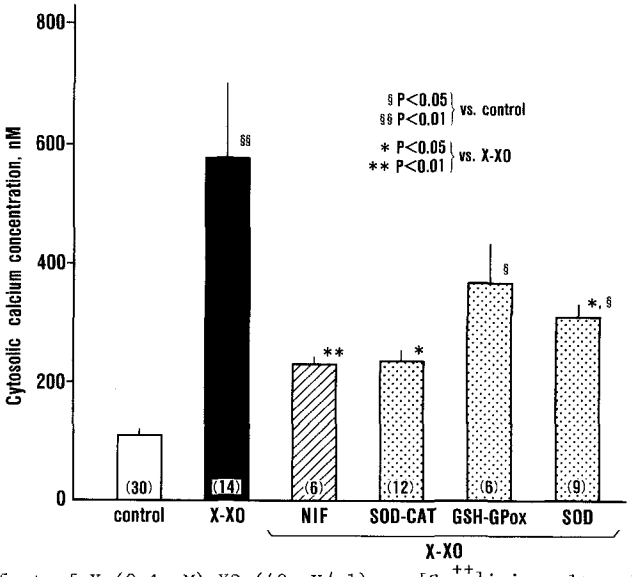

Fig. 1: Effect of X (0.1 mM)-XO (40 mU/ml) on $[Ca^{++}]i$ in cultured endothelial cells. Effects of nifedipine (NIF: $3.2x10^{-6}$M), SOD (100 μg/ml)-catalase (CAT: 100 μg/ml), GSH (10 μM)-GPox (10 mU/ml), and SOD (100 μg/ml) on X-XO-induced increase in $[Ca^{++}]i$ are also shown. Number of experiments are shown in parentheses.

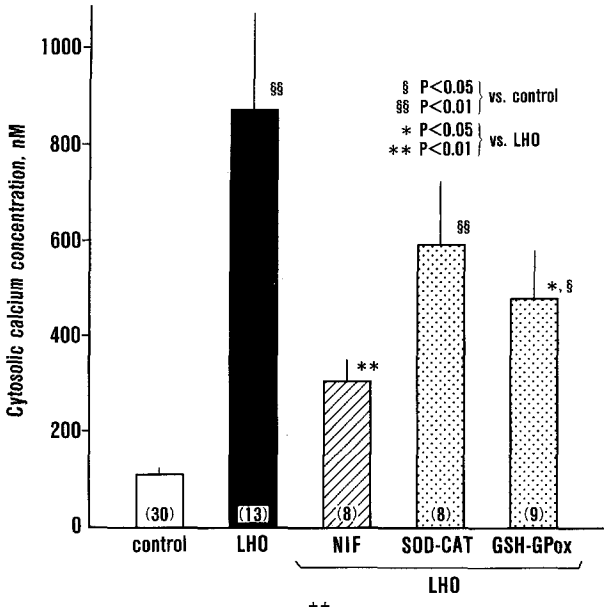

Fig. 2: Effect of LHO (0.32 μM) on $[Ca^{++}]i$ in cultured endothelial cells. Effects of nifedipine (NIF), SOD-catalase (CAT), and GSH-GPox on LHO-induced increase in $[Ca^{++}]i$ are also shown. Doses employed are the same as in Fig. 1. Number of experiments are shown in parentheses.

DISCUSSION

The present study demonstrated that both LHO and X-XO increased $[Ca^{++}]i$ in cultured endothelial cells. It is unlikely that this effect was due to non-specific cellular damage, since the cellular damage caused by LHO

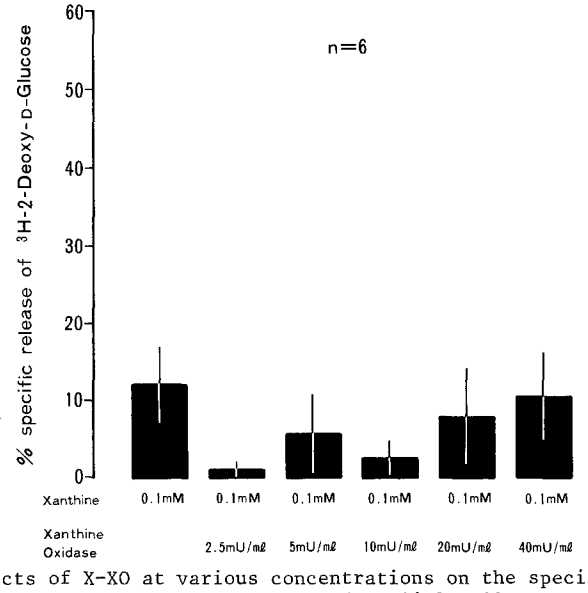

Fig. 3: Effects of X-XO at various concentrations on the specific release of $[^3H]$2-deoxy-D-glucose from cultured endothelial cells.

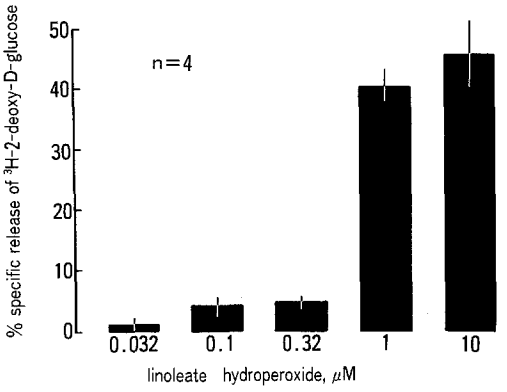

Fig. 4: Effects of LHO at various concentrations on the specific release of [^{3}H]2-deoxy-D-glucose from cultured endothelial cells.

and X-XO was not significant in the doses employed in this study. X-XO is known to generate superoxide radicals and LHO is also a member of reactive oxygen spieces. The effect of X-XO and LHO on [Ca^{++}]i was suppressed by the pretreatment with the respective scavenger enzyme, SOD and GSH-GPox , indicating that the effect is mediated by reactive oxygen. The effects were not definitive, however, of SOD-catalase on the LHO-induced increase in [Ca^{++}]i, and of GSH-GPox on the X-XO-induced increase in [Ca^{++}]i. These results may be explained by the fact that scavenging systems responsible for the elimination of superoxide and lipid peroxide are different (14-16). The other product from X-XO is hydrogen peroxide. Since SOD alone significantly inhibited the increase in [Ca^{++}]i provoked by X-XO, the contribution of hydrogen peroxide to the effect of X-XO might be minimal.

The role of calcium in the generation of superoxide in human leucocytes stimulated by concanavalin A (17) and by chemotactic peptides has been reported (18). However, the involvement of cytosolic calcium in the action of reactive oxygen has remained unknown. Shasby et al. have shown by using radioactive calcium that X-XO increases albumin transfer across cultured endothelial cells and cytosolic calcium is involved in the phenomenon (4). Bellomo et al. also reported that disturbance of calcium homeostasis is involved in the mechanism of cytotoxity evoked by lipid hydroperoxide (19). However, direct measurement of cytosolic calcium concentration has not been performed. To our knowledge, this is the first report that shows reactive oxygen increases [Ca^{++}]i in cultured endothelial cells.

Reactive oxygen spieces are known to be a risk factor for the development of atherosclerosis. These molecules provoke many adverse biological effects on endothelial cells such as morphological alteration (20-22), impairment of prostacyclin production (23) and the increase of

vascular permeability (4,24), all of which could promote atherosclerotic process. The present study indicates that cytosolic calcium might mediate , at least in part, these biological effects of reactive oxygen spieces.

We further revealed that calcium channel blocker, nifedipine, also suppressed the increase in $[Ca^{++}]i$ provoked by reactive oxygen. Since nifedipine acts mainly on voltage-dependent calcium channels (11), calcium might be mobilized through voltage-dependent calcium channels in ECs by the reactive oxygen spieces. Recent investigations have suggested the inhibitory effect of calcium antagonists on the development of atherosclerosis (25). The postulated mechanisms include the increase in prostacyclin production in vascular endothelial cells (26), the inhibition of platelet aggregation (27) and the inhibition of the proliferation of vascular smooth muscle cells (28). The results demonstrated in the present study suggest a new mechanism by which calcium antagonists exert the anti-atherogenic action.

ACKNOWLEDGEMENT

The authors would like to thank Drs. Tetsu Yamane and Youji Yoshida of the First Department of Pathology, Yamanashi Medical University, Kofu, Japan, for their kind suggestions concerning the endothelial cell culture. This study is supported by a Grant-in-aid for Scientific Research (No. 61480247) from the Ministry of Education, Science and Culture of Japan and a grant from Takeda Medical Foundation.

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