Oxidant-induced arachidonic acid release and impairment of fatty acid acylation in vascular smooth muscle cells

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Cane, Agnès, Michelyne Breton, Kamen Koumanov, Gilbert Béréziat, and Odile Colard. Oxidant-induced arachidonic acid release and impairment of fatty acid acylation in vascular smooth muscle cells. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C1040–C1046, 1998.—Oxidative damage, which plays a major role in the early stages of atherosclerosis, is associated with arachidonic acid (AA) release in vascular smooth muscle cells (VSMC) as in other cell types. In this study, H_2O_2 was used to investigate mechanisms of AA release from VSMC on oxidative stress. Cell treatment with H_2O_2 inhibited AA incorporation in an inverse relationship to prolonged H_2O_2-induced AA release. Identical kinetics of inhibition of AA incorporation and AA release were observed after cell treatment with AlF_4^-, a process not involving phospholipase A_2 (PLA_2) activation as recently described (A. Cane, M. Breton, G. Béréziat, and O. Colard. *Biochem. Pharmacol.* 53: 327–337, 1997). AA release was not specific, since oleic acid also increased in the extracellular medium of cells treated with H_2O_2 or AlF_4^- as measured by gas chromatography-mass spectrometry. In contrast, AA and oleic acid cell content decreased after cell treatment. Oleoyl and arachidonoyl acyl-CoA synthases and acyltransferases, assayed using a cell-free system, were not significantly modified. In contrast, a good correlation was observed between decreases in AA acylation and cell ATP content. The decrease in ATP content is only partially accounted for by mitochondrial damage as assayed by rhodamine 123 assay. We conclude that oxidant-induced arachidonate release results from impairment of fatty acid esterification and that ATP availability is probably responsible for free AA accumulation on oxidative stress by preventing its reesterification and/or transmembrane transport.

hydrogen peroxide; aluminum fluoride; cell adenosine triphosphate content; A_7r_5 cells

ARACHIDONIC ACID (AA) is an important cellular mediator acting directly and after transformation to oxidized products. Metabolites of AA play important roles in regulating early growth-response genes (20) and vascular tone (22, 32) in vascular smooth muscle cells (VSMC). Recent studies have suggested additional roles for unmetabolized AA in cell signaling, such as stimulation of tyrosine-containing protein phosphorylation (5), inhibition of myosin light chain phosphatase (13), and recovery of cell growth after arrest by depletion of the Ca^{2+} pool (14). The cellular level of unesterified AA depends on the relative activities of enzymes that catalyze AA hydrolysis from lipids and enzymes that catalyze AA reesterification (10, 16). A major pathway for the deacylation of AA from the *sn*-2 position of phospholipids involves cytosolic phospholipase A_2 (PLA_2) activation (11). Acyl-CoA synthesis in the presence of ATP is necessary for free AA acylation by acyltransferase into phospholipids.

Oxidative damage is a major pathophysiological event in a broad range of inflammatory states, including the early stages of atherosclerosis (22). H_2O_2 and oxygen metabolites were shown to trigger AA release and metabolism in cultured cells (4, 8, 12, 25, 26), including smooth muscle cells (7, 21). Whereas cytosolic PLA_2 activation has been observed after H_2O_2 treatment in some cell systems (7, 8, 21), the mechanism of AA release appears to be independent of Ca^{2+} in other cell systems (4, 12). Sporn et al. (25) showed that oxidative stress induced by H_2O_2 treatment of alveolar macrophages resulted in AA release by inhibition of AA esterification into phospholipids in association with depletion of ATP.

Recently, we characterized a Ca^{2+}-independent process of AA release in A_7r_5 VSMC (6). Indeed, VSMC triggering by the direct G protein activator AlF_4^- induced a slow and linear release of AA that was not accompanied by Ca^{2+} mobilization or Ca^{2+} entry into the cells. This AA release did not involve any known phospholipase A_2, in contrast to the rapid and Ca^{2+}-dependent release of AA induced by vasopressin that mobilizes Ca^{2+} and translocates cytosolic PLA_2. It was then of interest to investigate whether the process of AA release from VSMC treated with H_2O_2 was PLA_2 dependent or independent. We show that AlF_4^- and H_2O_2 induced an AA release that was inversely related to the acylation rate of fatty acids and ATP content of VSMC.

METHODS

Cell culture. Rat aortic smooth muscle cells (A_7r_5) were obtained from European Collection Animal Cell Cultures. The cells were grown at 37°C in DMEM supplemented with 10% (vol/vol) FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-FCS) under a 5% CO_2 atmosphere. A_7r_5 cells were subcultured every 7 days using trypsin-EDTA.

AA release. Confluent cells in 35-mm dishes were labeled for 1 h with 0.4 μCi of [^{3}H]AA per dish in 1 ml of DMEM-FCS. Alternatively, cells were labeled overnight in the same conditions. The medium was changed, and H_2O_2 (200 μM) or AlF_4^- (5 mM NaF + 10 μM $AlCl_3$) was added for various times. The supernatants were collected and counted for radioactivity by liquid scintillation.

AA incorporation. DMEM-FCS contained ~0.5 nmol/ml as measured by gas chromatography-mass spectrometry (GC-MS). AA incorporation was assayed in FCS-containing medium (0.5 μM AA) or in FCS plus 1.5 nmol AA/ml (2.0 μM AA). [^{3}H]AA was present in the incubation medium such that AA specific activity was 0.8 μCi/nmol in both cases. H_2O_2 or AlF_4^-

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was added together with [^{3}H]AA. Supernatants were discarded, and cells were washed and scraped in 1:0.8 (vol/vol) methanol-H_2O. Lipids were extracted by addition of chloroform and methanol (2), the organic phase was evaporated under N_2, and the radioactivity was counted.

ATP assay. Cellular ATP was determined by the luciferase-luciferin assay. After cell incubation, culture media were removed and cells were washed twice with ice-cold PBS. They were then scraped into 1 ml of 10 mM KH_2PO_4-4 mM $MgSO_4$ buffer, pH 7.4 (*buffer A*), and transferred to ice. Cell suspensions were placed in a 90–95°C water bath for 4 min and then on ice until assay. Twenty microliters of sample diluted in 1 ml of *buffer A* were added to 2 ml of 50 mM Na_2HAsO_4-20 mM $MgSO_4$ buffer, pH 7.4 (*buffer B*). Fifty microliters of luciferase-luciferin reconstituted in sterile water (20 mg/ml) were added to the assay mixture, and chemiluminescence was measured over 10 s in a Lumat LB 9501 apparatus (Berthold).

Rhodamine 123 uptake. The changes in mitochondrial membrane potential were evaluated by measuring the cellular retention of rhodamine 123 (17). Cells were incubated for 30 min at 37°C with 5 μM rhodamine 123, then washed and allowed to stand at 37°C for 45 min in a rhodamine-free medium. After removal of the medium, cells were treated with agonists for 2 h. The dye trapped in treated and nontreated cells was determined by fluorometric analysis after lysis in 1% Triton. The excitation wavelength was 490 nm, and the emission wavelength was 515 nm.

Thiazolyl blue assay. Cellular reductive capacity was assayed by reduction of tetrazolium salt [3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazolium bromide (MTT)] (24). Cells were incubated for 4 h at 37°C with MTT (50 μg/ml) and then washed and treated with agonists for 2 h. Control and treated cells were lysed in 5% SDS, and the absorbance of the MTT reduced form was read at 540 nm.

Neutral red retention. Cell viability was evaluated by measuring the cellular retention of neutral red (3). Controls and cells triggered with AlF_4^- or H_2O_2 for 2 h were loaded for 3 h at 37°C with medium containing 0.05% neutral red. After cell lysis in 5% SDS, the absorbance of neutral red trapped in cells was read at 535 nm.

Acyl-CoA synthase assay. Arachidonoyl-CoA synthase and oleoyl-CoA synthase were assayed as described by Wilson et al. (31). Control and treated cells were sonicated in 50 mM Tris·HCl, pH 8.0, containing phenylmethylsulfonyl fluoride (20 μg/ml). The incubation medium consisted of 20 mM $MgCl_2$, 6.7 mM ATP, 0.7 mM CoA, 1 mM β-mercaptoethanol, 2 mM Triton X-100, 100 μM fatty acid [a mixture of unlabeled AA or oleic acid (OA) and 30 nCi of [1-^{14}C]AA (55 mCi/mmol; Amersham) or 30 nCi of [9,10-^{3}H]OA (10 Ci/mmol; Dupont-New England Nuclear)], and homogenate in a total volume of 150 μl of Tris buffer, pH 8.0. After incubation at 37°C for 10 min, the reaction was terminated by addition of 2.25 ml of 40:10:1 (vol/vol/vol) isopropanol-heptane-H_2SO_4 (2 M); 1.5 ml of heptane and 1 ml of water were then added, and the mixture was vortexed. The aqueous phase containing the radiolabeled acyl-CoA formed was washed twice with 2 ml of heptane, and radioactivity was determined by scintillation counting.

Lysophosphatidyl acyltransferase assay. Arachidonoyl-CoA lysophosphatidylcholine (LPC) acyltransferase and oleoyl-CoA LPC acyltransferase were assayed. Control and treated cells were sonicated in 140 mM KCl and 20 mM HEPES, pH 7.4, containing 20 μg/ml phenylmethylsulfonyl fluoride. The reaction mixture contained 20 μM arachidonoyl-CoA or oleoyl-CoA, 32 μM LPC [a mixture of unlabeled LPC and 10 nCi of L-1-[palmitoyl-1-^{14}C]LPC (56 mCi/mmol; Amersham)], 0.2% BSA, 140 mM KCl, and homogenate (20–40 μg) in a total volume of 250 μl of HEPES buffer, pH 7.4. The incubation was performed at 37°C for 10 min, the reaction was stopped by addition of chloroform-methanol, and the lipids were extracted according to Bligh and Dyer (2). Extracted lipids were subjected to TLC on silica gel plates using 50:25:8:4 (vol/vol/vol/vol) chloroform-methanol-acetic acid-water. The LPC and phosphatidylcholine bands, visualized with I_2 vapor, were scraped, and radioactivity was counted.

Fatty acid analysis by GC-MS. Cells were incubated for 2 h in DMEM-FCS containing AlF_4^- or H_2O_2. The free fatty acids, from supernatants and attached cells scraped in 1 ml of cold PBS, were extracted with 5 vol of 10:40:0.1 isopropanol-hexane-H_2SO_4 and methylated with diazomethane. They were then separated by GC on a capillary column containing Supelcowax 10 bonded phase (0.32 mm diameter, 30 m long) on a Hewlett-Packard 5890 series II gas chromatograph. Fatty acids were detected by MS (model R10-10C, Nermag) in the chemical ionization mode with ammonia (0.1 bar) as the reagent gas. The positive quasi-molecular ions were monitored and time integrated. Quantification was referred to heptadecanoic methyl ester as an internal standard, and the response factors of the fatty methyl esters were calibrated for each experiment.

RESULTS

We first compared kinetic curves of AA release, induced by 200 μM H_2O_2 or 10 μM AlF_4^-, from VSMC prelabeled with [^{3}H]AA for 60 min (Fig. 1). The release of AA from cells exposed to 10 μM AlF_4^- for 2 h represented ~20% of lipid labeling (6). The release of AA induced by 200 μM H_2O_2 was in the same range. This noncytolytic concentration was then chosen to compare kinetic curves. Both compounds induced, after a 30-min latency period, a linear AA release up to 120 min representing 21.6 ± 2.8 and 16.8 ± 3.2% after treatment with AlF_4^- and H_2O_2, respectively. Linear release after the latency period was also observed after overnight prelabeling. AA release after overnight prelabeling was somewhat lower than after 1 h of labeling in control and stimulated cells, such that the ratio of stimulated to control cells remained constant (Table 1).

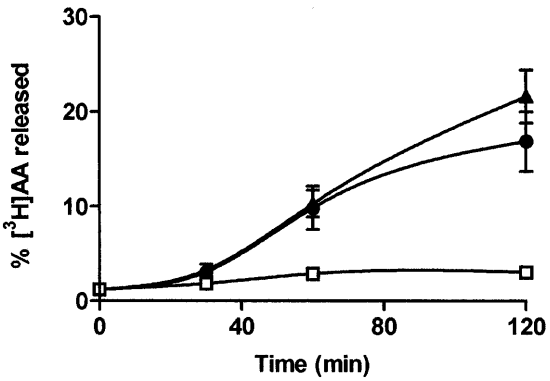

Fig. 1. Free arachidonic acid (AA) release from vascular smooth muscle cells (VSMC) triggered by AlF_4^- and H_2O_2. Cells prelabeled with 0.4 μCi of [^{3}H]AA for 60 min were incubated in DMEM-FCS (□), AlF_4^- (5 mM NaF + 10 μM $AlCl_3$, ▲), or 200 μM H_2O_2 (●) for various periods of time. [^{3}H]AA released in medium is expressed as percentage of total radioactivity (cell + medium). Values are means ± SE of 8 determinations.

Table 1. *AA release after 1 or 16 h of prelabeling*

Treatment	%AA Release	
	1 h	16 h
None	4.3 ± 1.2	2.9 ± 0.7
AlF_4^-	14.6 ± 0.9 (3.4)	10.3 ± 1.4 (3.5)
H_2O_2	12.5 ± 1.5 (2.9)	8.0 ± 0.3 (2.7)

Values are means ± SD of 2 determinations. Values in parentheses represent magnitude increase relative to nonstimulated cells. Cells prelabeled with 0.4 μCi of ^{3}H-labeled arachidonic acid (AA) for 1 or 16 h were incubated as described in Fig. 1 legend for 90 min.

This finding favors involvement of the same lipid pools in AA release observed in the two prelabeling conditions. The kinetics of AA release after AlF_4^- and H_2O_2 treatment were then similar and completely different from vasopressin-mediated AA release, which resulted in Ca^{2+} mobilization and cytosolic PLA_2 translocation (6).

If regular and long-lasting appearance of AA in the extracellular medium does not depend on phospholipid hydrolysis, it can be the consequence of decreased reacylation mechanisms. Therefore, we measured the effect of both agonists on the rate of AA incorporation (Fig. 2). In control cells, incorporation of [^{3}H]AA into lipids was nearly linear up to 120 min, where it represented 25% of initial radioactivity. Addition of AlF_4^- or H_2O_2 together with [^{3}H]AA resulted in a decrease in AA incorporation at 30–60 min followed by a complete inhibition of the incorporation. H_2O_2 had slightly less effect than AlF_4^- on AA incorporation as on AA release (Figs. 1 and 2). Triacylglycerols and phospholipids were separated on TLC in two experiments. The AA incorporation into triacylglycerols remained very low (2.6 ± 0.6%) in control and treated cells. Thus the decrease in AA acylation of treated cells relative to control cells was essentially due to decreased AA incorporation into phospholipids.

It remained a possibility that a decrease in labeled AA incorporation might be observed if the treatments led to a decrease in AA specific activity. Such would be the case if the treatments led to an increase in fatty acid deacylation, thus enhancing AA concentration. Therefore, we incorporated AA at two different concentrations. As shown in Fig. 3, the amount of AA incorporated into lipids of control and stimulated cells was increased by 3.4 ± 0.2-fold in the presence of 2 μM AA compared with 0.5 μM in the control medium. The decrease in AA incorporation when cells were incubated with AlF_4^- or H_2O_2 was in the same range whether or not 1.5 μM AA was added to FCS-containing medium. This experiment demonstrates that an increase in AA deacylation, because it enhanced AA concentration, would not decrease AA incorporation and rules out the possibility that decreased AA incorporation was due to increased AA deacylation.

The accumulation of free fatty acids in the extracellular medium was then measured by GC-MS (Table 2). In two separate experiments, AA increased from 529 ± 41 pmol in supernatants from control cells to 730 ± 106 and 681 ± 98 pmol when ^{5} cells were treated with AlF_4^- and H_2O_2, respectively. To investigate whether this increase was specific for AA, OA content in the medium was also examined. The medium had a much higher content of OA (4,079 ± 453 pmol/ml) than of AA. Cell treatment also increased the amount of OA in the extracellular medium. Surprisingly, the ratio of the amount of free fatty acids in cells and medium (1:20) was in the same range for OA and AA. There was no accumulation, rather there was a decrease, of the intracellular content of either fatty acid after exposure to agonists.

Fatty acid incorporation into phospholipids requires two enzymatic activities: an acyl-CoA synthase and an acyl-CoA acyltransferase. If one of these steps is altered, free fatty acids should accumulate in the medium. We assayed the enzyme activities with OA and AA in sonicated cells (Table 3). The oleoyl transferase activity was considerably higher, ~10-fold, than the arachidonoyl transferase activity, whereas oleoyl-CoA

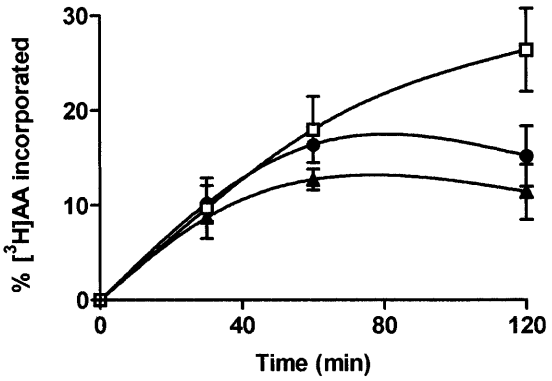

Fig. 2. AlF_4^- and H_2O_2 inhibited AA incorporation in VSMC. Cells were incubated in DMEM-FCS containing 0.4 μCi of [^{3}H]AA without (□) or with AlF_4^- (▲) or H_2O_2 (●). After incubation for different periods of time, supernatant was discarded and cell lipids were extracted. [^{3}H]AA incorporated into cell lipids was counted and expressed as percentage of initial radioactivity. Values are means ± SE of results from 6 experiments.

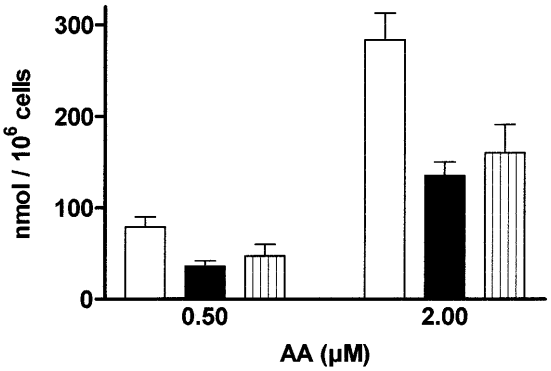

Fig. 3. Inhibition of AA incorporation did not depend on AA concentration. Cells were incubated in DMEM-FCS containing 0.5 or 2.0 μM AA ([^{3}H]AA, sp act = 0.8 μCi/nmol) without (open bars) or with AlF_4^- (filled bars) or H_2O_2 (striped bars). After 90 min of incubation, sample cells were treated as described in Fig. 2 legend. Data are expressed as pmol AA incorporated per 10^5 cells. Values are means ± SE of 4 different experiments.

Table 2. *Free fatty acid mass content*

	Control	AlF_4^-	H_2O_2
AA (20:4)			
Supernatants	529 ± 41	730 ± 106	681 ± 98
Cells	37 ± 9	24 ± 9	30 ± 10
OA (18:1)			
Supernatants	4,079 ± 453	4,941 ± 607	4,930 ± 462
Cells	367 ± 83	297 ± 115	212 ± 20

Values (pmol/well) are means ± SE of 2 experiments performed in duplicate ($n = 4$). Cells (10^5/well) incubated in 1 ml of DMEM-FCS were triggered without (control) or with AlF_4^- or H_2O_2 for 2 h. Free fatty acids were extracted from supernatants and cell pellets and analyzed by gas chromatography-mass spectrometry. Heptadecanoic acid (17:0) was added as an internal standard.

synthesis was only twice as high as arachidonoyl-CoA synthesis. In our incubation conditions, no significant change in these different enzyme activities was observed after cells were treated with AlF_4^- or H_2O_2.

It then appears that decreased AA incorporation is not accounted for by inhibition of enzyme activities. Because ATP is necessary for acyl-CoA synthase (18) and H_2O_2 decreased ATP content in alveolar macrophages (27), we measured cell ATP content. As shown in Fig. 4, the ATP content declined by one-half in cells treated with AlF_4^- for 2 h. Treatment with H_2O_2 diminished the ATP content slightly less than treatment with AlF_4^-. These decreases in ATP content were well correlated with the inhibition of AA acylation (Fig. 5).

Inasmuch as mitochondria represent a major source of ATP and were proposed to be the primary target of H_2O_2 action in neuronal cells (30), we investigated the effect of AlF_4^- or H_2O_2 on the integrity of mitochondria using rhodamine 123 assay (Table 4). Rhodamine 123 is selectively taken up by mitochondria and then reflects maintenance of the mitochondrial potential ($\Delta\Psi$) (17). Exposure of cells to AlF_4^- or H_2O_2 for 2 h slightly reduced the retention of rhodamine 123 by mitochondria. We also used the MTT assay to evaluate the reductive capacity of cells (24). The assay is based on the ability of the cell to produce formazan (reduced

Table 3. *Effect of* AlF_4^- *and* H_2O_2 *on enzymes involved in AA incorporation*

	CoA Synthase		Acyltransferase	
Treatment	A-CoA S (20:4)	O-CoA S (18:1)	A-AT (20:4)	O-AT (18:1)
None	100	100	100	100
AlF_4^-	93.0 ± 7.2	87.5 ± 5.4	109.0 ± 11.2	99.2 ± 2.8
H_2O_2	103.7 ± 11.4	102.1 ± 10.1	105.0 ± 4.7	94.5 ± 2.0

Values are means ± SE expressed as a percentage of activities in control homogenates from unexposed cells. Cells were incubated for 2 h in media containing AlF_4^- (5 mM NaF + 10 μM $AlCl_3$) or H_2O_2 (200 μM), then scraped into homogenizing buffer and sonicated. Homogenates were assayed for arachidonoyl- and oleoyl-CoA synthase activities (A-CoA S and O-CoA S) and for arachidonoyl- or oleoyl-CoA acyltransferase activities (A-AT and O-AT). Activity of each enzyme in control homogenates was as follows: 5,712 ± 349 (A-CoA S, $n = 8$), 4,109 ± 296 (O-CoA S, $n = 8$), 409 ± 12 (A-AT, $n = 8$), and 9,080 ± 189 (SE) pmol · mg protein^{-1} · min^{-1} (O-AT, $n = 8$).

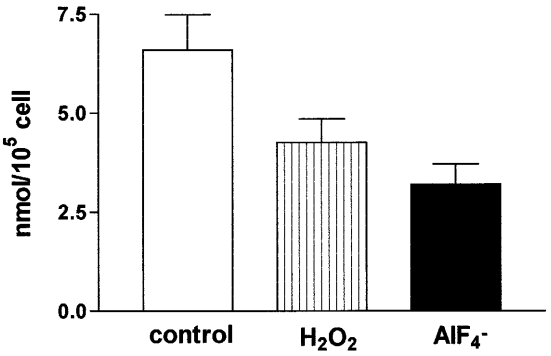

Fig. 4. Decrease in ATP content of cells triggered with AlF_4^- and H_2O_2. Cells were incubated in 10% DMEM-FCS with agonists for 2 h. Culture medium was removed, cells were harvested, and their ATP content was determined by luciferase-luciferin assay. Values are means ± SE of 6 determinations.

form of MTT). No inhibitory effect of AlF_4^- or H_2O_2 was observed on this enzyme activity. Finally, neutral red was used to evaluate the cytotoxic effect of treatments on membrane structures (3) (Table 4). Control or treated cells were incubated for 2 h with the dye, and the neutral red trapped in the cells was measured. Again, there was no significant difference between treated and nontreated cells. The two last assays demonstrated that treatment of cells with H_2O_2 or AlF_4^- for 2 h did not alter cell viability.

DISCUSSION

H_2O_2 is a major oxygen metabolite produced by activated inflammatory cells, including macrophages and neutrophils (23). These cells, together with T lymphocytes, are present in the intima at the early stages of atherosclerosis (22). H_2O_2 produced by these inflammatory cells can thus be in contact with VSMC. In this study we investigated the mechanisms involved in H_2O_2-induced AA release from VSMC. Our data strongly suggest that oxidant-induced AA release from VSMC does not depend on PLA_2 activation but results from impairment of fatty acid reesterification. First, incorporation of [^{3}H]AA into cell phospholipids was

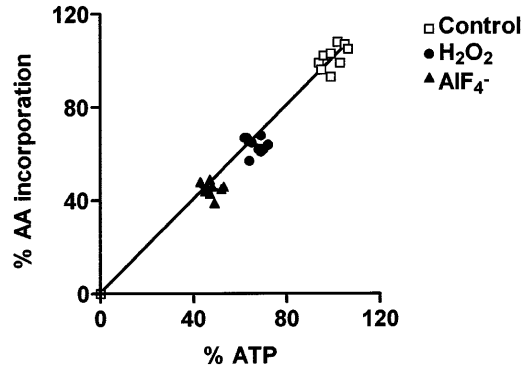

Fig. 5. Correlation between cell ATP content and AA incorporation in VSMC. Data are from Figs. 2 and 3. Mean values for ATP and AA esterification in control cells were taken as 100. AA incorporation and ATP content in control and treated cells for each different experiment were then expressed as percentage of this value.

Table 4. *AlF_4^- and H_2O_2 only slightly affected mitochondrial metabolic activity*

Treatment	Neutral Red ($n=3$)	Rhodamine 123 ($n=5$)	MTT ($n=8$)
None	100	100	100
AlF_4^-	103.6 ± 5.7	85.1 ± 8.4	104.8 ± 3.6
H_2O_2	105.3 ± 1.0	89.1 ± 6.6	98.1 ± 3.2

Values are means ± SE expressed as a percentage of values in control conditions. Cells were triggered with agonist for 2 h after or before loading with 5 μM rhodamine 123, 50 μg/ml MTT, or 0.05% neutral red. After incubation, supernatants were discarded, cells were lysed, and respective absorbances were read. For each test, cells from duplicate 6-well plates were read for absorbance values or used for protein.

inhibited in an inverse way to AA release from prelabeled cells on exposure to H_2O_2. Inhibition of AA esterification cannot be accounted for by a decrease in AA specific activity due to increased deacylation, since inhibition rates were similar in the presence of 0.5 and 2.0 μM AA. Moreover, increasing AA concentration considerably enhances its incorporation. Second, inhibition of AA incorporation together with AA release was also observed after treatment of cells with AlF_4^-, a process not involving PLA_2 activation (6). Third, AA release was not specific, since OA also increased in the extracellular medium of cells treated with H_2O_2 or AlF_4^-, as demonstrated by GC-MS. These data are not consistent with the liberation of AA by an arachidonate-specific PLA_2 (11).

Oxygen species have been shown to trigger AA release in a number of cell types (4, 8, 12, 25), including VSMC (7, 21). A role for cytosolic PLA_2 has often been suggested but not clearly demonstrated. Increased synthesis of cytosolic PLA_2 is indeed involved in AA release after ultraviolet B injury of keratinocytes (8), among many other long-term effects. In growth-arrested VSMC, H_2O_2 induced very slight phosphorylation of cytosolic PLA_2 and mitogen-activating protein kinase (21), which could not completely account for the large AA release observed. In endothelial cells, involvement of a PLA_2 specific for arachidonate was deduced from the absence of [^{3}H]OA release from prelabeled cells treated with H_2O_2 (4). However, we observed an accumulation of OA as well as AA in the medium of VSMC treated with H_2O_2.

On the other hand, Sporn et al. (25) demonstrated that, in alveolar macrophages, H_2O_2 increased the availability of AA by inhibiting its acylation into phospholipids. Our data are in agreement with their results. AA esterification is inhibited in our VSMC cultured in 10% FCS and treated with H_2O_2 or AlF_4^-. Moreover, not only AA but also OA accumulates in the medium. It is then likely that esterification of all fatty acids is prevented. In addition, cell content in free fatty acids decreased on oxidative stress, despite their accumulation in the medium. This impairment of fatty acid esterification could be a general process by which H_2O_2 resulted in free AA accumulation. Continual fatty acid remodeling between cell phospholipids (10) implicates basal PLA_2 activity together with acyltransferase and transacylase activities. Part of the cytosolic PLA_2 appears to be constitutively bound to membranes of resting cells, as observed in our VSMC line (6), as in other cells (11), and could account for fatty acid remodeling and basal release. This basal release would be responsible for fatty acid accumulation when fatty acid reesterification is prevented.

In a second set of experiments we investigated the mechanism of the impairment of fatty acid esterification. We first assayed, in a cell-free system, the two enzyme activities necessary for free fatty acid incorporation into phospholipids: the acyl-CoA synthase, which forms acyl-CoA complex from free fatty acid, CoA, and ATP, and the acyl-CoA acyltransferase, which incorporates the fatty acyl moiety of acyl-CoA into a lysophospholipid (18). When saturating concentrations of the various substrates and cofactors were used, the oleoyl transferase was considerably higher than the arachidonoyl transferase. In contrast, arachidonoyl and oleoyl acyl-CoA synthase activities were in the same range. Neither of these activities was decreased when cells were pretreated with H_2O_2 or AlF_4^- for 2 h. Because no change in these enzyme activities could explain the impairment of fatty acid esterification and because ATP is involved in acyl-CoA synthesis, we then measured cell ATP content. H_2O_2 and AlF_4^- treatment decreased cell ATP content by 36% and 50%, respectively. These decreases correlated well with the inhibition of AA esterification. Such a correlation between accumulation of free AA and depletion of ATP has been observed in cultured myocardial cells (9, 15) and in alveolar macrophages triggered with H_2O_2 (25). Muscle cells are known to contain a large amount of ATP, required essentially for muscle contraction-relaxation. The ATP content of our cell line, even after H_2O_2 or AlF_4^- treatment and a 50% decrease in ATP, is indeed ~100-fold higher than the ATP content of alveolar macrophages (27). We could wonder whether such a decrease in ATP induced by agonists would be sufficient to prevent fatty acid reesterification. However, despite the relatively high amount of ATP remaining in cells after treatment with the agonists, a good correlation was observed between ATP content and AA esterification. Then it is likely that different ATP pools exist in muscle cells and that the pool used for acyl-CoA synthesis is sufficiently decreased to prevent acyl-CoA formation. Alternatively, membrane proteins that might be involved in the transmembrane transport of fatty acids were recently discovered (29), discrediting the hypothesis that fatty acids crossed the membrane by simple diffusion. Because free fatty acid cell content slightly decreased after H_2O_2 or AlF_4^- treatment, the transmembrane transport of fatty acids appears somewhat altered by oxidative stress and might also be involved in the decrease of reesterification.

Because activities of mitochondria have been suggested in various models of oxidative stress to represent a major target for prooxidant molecules (28, 30)

and mitochondria represent a source of ATP, we investigated the action of AlF_4^- and H_2O_2 on cell viability and integrity of mitochondria. Cell viability evaluated by neutral red uptake or the MTT assay was not significantly modified by 2 h of treatment with agonists, whereas mitochondrial potential, as assayed by rhodamine 123, was slightly affected. However, it is likely that these low reductions, which appear not to be comparable to the marked decrease in ATP content, account only partially for the decrease in ATP content.

AA esterification in pancreatic islets was impaired by an inhibitor of ATP synthase (19). Changes in enzyme activities involved in ATP synthesis might then account for the decrease in cell ATP content observed after treatment with H_2O_2 or AlF_4^-. Moreover, Balsinde et al. (1) found that cholera and pertussis toxins had no effect on AA incorporation into phospholipids in mouse peritoneal macrophages, implying that G proteins do not regulate acylation in these cells. It is then likely that AlF_4^- depletes ATP and inhibits fatty acid acylation by a non-G protein-dependent mechanism.

In summary, our data demonstrate that oxidant-induced AA release from VSMC results from impairment of fatty acid reesterification and that this prevention in esterification may be due to a decrease in cell ATP content, which appears to be provoked only partially by damage to the mitochondria. The transmembrane transport of fatty acids might also be involved in the diminution of fatty acid esterification. The level of ATP, free AA, and other fatty acids, depending on oxidative stress, could then play an important role in atherosclerosis.

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