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Aldosterone Up-Regulates 12- and 15-Lipoxygenase Expression and LDL Oxidation in Human Vascular Smooth Muscle Cells

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ABSTRACT

Several lines of evidence suggest that aldosterone excess may have detrimental effects in the cardiovascular system, independent of its interaction with the renal epithelial cells. Here we examined the possibility that aldosterone modulates 12- and/or 15-lipoxygenase (LO) expression/activity in human vascular smooth muscle cells (VSMC), in vitro, thereby potentially contributing to both vascular reactivity and atherogenesis. Following 24 h treatment of VSMC with aldosterone (1 nmol/L), there was a ~2-fold increase in the generation rate of 12 hydroxyeicosatetraenoic acid (12-HETE), 70% increase in platelet type 12-LO mRNA expression ($P < 0.001$) along with a ~3-fold increase in 12-LO protein expression, which were blocked by the mineralocorticoid receptor (MR) antagonists spironolactone (100 nmol/L) and eplerelone (100 nmol/ml). Additionally, aldosterone (1 nmol/L; 24 h) increased the production of 15-HETE (50%; $P < 0.001$) and the expression of 15-LO type 2 mRNA (50%; $P < 0.05$) (in VSMC). Aldosterone also increased the 12- and 15-LO type 2 mRNA expression in a line of human aortic smooth muscle cells (T/G HA-VSMC) (60% and 50%, respectively). Aldosterone-induced 12- and 15-LO type 2 mRNA expressions were blocked by the EGF-receptor antagonist AG 1478 and by the MAPK-kinase inhibitor UO126. Aldosterone-treated VSMC also showed increased LDL oxidation, (~2-fold; $P < 0.001$), which was blocked by spironolactone. In conclusion, aldosterone increased 12- and 15-LO expression in human VSMC, in association with increased 12- and 15-HETE generation and enhanced LDL oxidation and may directly augment VSMC contractility, hypertrophy, and migration through 12-HETE and promote LDL oxidation via the pro-oxidative properties of these enzymes. J. Cell. Biochem. 108: 1203–1210, 2009.

KEY WORDS: ALDOSTERONE; VASCULAR SMOOTH MUSCLE CELL; 12-LIPOXYGENASE; 15-LIPOXYGENASE; LDL OXIDATION

Although aldosterone interacts with specific soluble intracellular mineralocorticoid receptors (MR) widely expressed in the kidney, colon, lung, salivary gland, heart, vasculature, and central nervous system, its major effects on cardiovascular function and disease have been traditionally attributed to the promotion of sodium retention and potassium excretion in the kidney [Funder, 1997; Farman and Rafestin-Oblin, 2001; Lifton et al., 2001]. Recent studies suggest, however, an array of formerly unrecognized effects of aldosterone within the cardiovascular system per se which appear to contribute significantly to cardiovascular pathology [Fritsh Neves and Schiffrin, 2003; Liu et al., 2003]. High circulating aldosterone is associated with endothelial dysfunction, enhanced vascular reactivity, and increased large artery rigidity [Brilla et al., 1993; Hatakeymana et al., 1994; Liu et al., 2003]. High serum aldosterone predicts increased mortality in patients with congestive heart failure [Swedberg et al., 1990] and is a marker for subsequent evolution of hypertension in otherwise normal individuals [Vasan et al., 2004]. Concordant with these findings, the use of low doses of the aldosterone antagonists spironolactone or eplerenone markedly reduced cardiovascular events in congestive heart failure [Pitt et al., 1999, 2003b], retarded renal injury in diabetes, [Hollenberg, 2004], and diminished left ventricular hypertrophy in essential hypertension [Pitt et al., 2003a]. Experimental evidence indicates that aldosterone excess is involved in coronary and myocardial inflammation and fibrosis [Brilla et al., 1993]. Several reports demonstrated that aldosterone affects vascular smooth muscle cells (VSMC) via direct interaction with MR, which are expressed in these cells [Mnegold et al., 1999; Jaffe and Mendelsohn, 2005].

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12- and/or 15-lipoxygenase (LO) are dioxygenase enzymes that incorporate molecular oxygen into unsaturated fatty acids, particularly arachidonic and linoleic acid, and are expressed in human VSMC [Limor et al., 2001]. LOs and their products play an important role in mediating LDL oxidation [Steinberg et al., 1989; Kuhn and Chan, 1997] and 12 hydroxyeicosatetraenoic acid (12-HETE) has been implicated in VSMC growth and migration, and in angiotensin II-dependent contraction [Stern et al., 1989; Kisch et al., 1997]. In the present study, we examined the possibility that aldosterone modulates 12- or/and 15-LO expression and activity in VSMC, thus, potentially contributing to vascular reactivity and atherogenesis through this system.

MATERIALS AND METHODS

MATERIALS

D-Aldosterone and spironolactone were purchased from Sigma–Aldrich Corporation, (St. Louis, MO). Antibodies for human platelet type 12-LO were generated in our laboratory as previously described [Limor et al., 2001]. AG1478, UO126, and PPI were purchased from Biomol (Plymouth Meeting, PA).

CELL CULTURE

VSMC were prepared from human umbilical arteries as previously described with minor modifications [Limor et al., 2001]. Cell migration was detected within 10–20 days. T/G HA-VSMC cell line was purchased from American Type Culture Collection (ATCC). Cells were trypsinized, transferred to 10 cm tissue culture dishes or six-well plates and cultured to subconfluence, at which time they were used for RNA and protein preparation, LDL oxidation and the measurement of 12- and 15-HETE. For all experiments only the first passage of cells was used.

TOTAL RNA PREPARATION AND PCR AMPLIFICATION

Before RNA preparation, cultured cells were incubated for 24 h in Medium 199, containing 0.4% FCS (in the absence of detectable steroids in the medium). Total RNA from cultured VSMC or T/G HA-VSMC was extracted with a mono-phasic solution of phenol and guanidine isothiocyanate (TRIZOL Reagent, Gibco, USA). Total RNA was quantified by spectrophotometry and evaluated on an agarose gel. One microgram of total RNA was reversed transcribed using a commercial method (Advantage RT for PCR, Clontech, Palo Alto, CA) and further PCR amplified. Real-time PCR was performed using assay on demand for human platelet type 12-LO on the real-time sequence detection system 7700 (Applied Biosystems, Foster City, CA).

All oligonucleotides were synthesized by Sigma and were purified by HPLC. The sequences of nucleotides were designed based on the reported sequences of the various genes, as previously outlined [Limor et al., 2001, 2005].

IMMUNOPRECIPITATION AND WESTERN IMMUNOBLOTTING

Cultured cells were washed with PBS, scraped and lysed on ice in a lysis buffer consisting of PBS (7.4) supplemented with 1% Triton X-100, one tablet of Boeringer Proteinase Inhibitors (Mannheim, Germany), and 0.1% SDS. This was followed by mild glass homogenization (10 strokes) and centrifugation at 10,000*g* for 10 min at 4°C. Aliquots of the supernatant were saved for protein estimation and Western blot analysis. Because of the low levels of the platelet-type 12-LO protein in VSMC, we also assessed its expression via immunoprecipitation. Lysates containing equal amounts of protein were incubated with 12-LO antibodies over night. The precipitates were further incubated with magnetic beads, washed with lysis buffer and then resuspended in SDS–PAGE sample buffer. After being denatured at 70°C for 10 min, samples were separated by SDS–PAGE and transferred to nitrocellulose membrane for immunoblotting.

For Western transfer of protein to Protean nitrocellulose (BA85) (S&S) standard methods were used. Immunoblots were developed with ECL or ECL plus (GE Healthcare, Amersham Biosciences, Buckinghamshire, England).

ACTIVATION OF MAPK

Cells were grown in six-well plates and serum-starved (0.1% FCS) for 16 h. Following treatment with the various agents, cells were washed twice with ice-cold PBS and were harvested in a lysis buffer (containing 40 mM HEPES, 100 mM NaCl, 1% Triton X-100, 1% Glycerol, 0.1 M EDTA, 40 mM NaF, 0.1 mM ammonium molybdate, 1 mM Na_3VO_4, 1 mM PMSF), followed by centrifugation (15,000*g*, 15 min, 4°C). The supernatants were collected and separated on 10% SDS–PAGE followed by Western blotting with human monoclonal anti-active MAPKs (ERK). Total MAPKs were detected with polyclonal antibodies as a control. The blots were developed with an HRP-conjugated secondary antibody in enhanced chemiluminescence's reaction.

LDL OXIDATION BY VSMC

VSMC were cultured in six-well plates and treated for 24 h with aldosterone (1 nM), in the absence and presence of spironolactone (0.01 μM). Afterwards the cells were incubated with LDL (100 μg/ml) in RPMI (phenol free) medium with $CuSO_4$ (2 μM) for 18 h and LDL oxidation was measured in the medium by a biochemical assay with thiobarbituric acid-reactive substances (TBARS) which is used to detect lipid peroxidation [Aviram and Vaya, 2001].

EXTRACTION AND MEASUREMENT OF 12- AND 15-HETE

Cultured confluent cells were washed twice with medium containing 0.4% FCS. Aldosterone and aldosterone antagonists were added for 24 h. The next morning the cells were washed, the antagonists were added for 1 h and aldosterone for another 30 min. The incubation was stopped by adding to the dishes 7.5 ml of ice-cold ethanol (100%; 0°C). For the measurement of 12- and 15-HETE, we used a modification of the reverse phase UV-HPLC method of Eskra et al. [1986], which has been validated by RIA, as previously described.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Data were assessed by Kruskal–Wallis non-parametric ANOVA test. Statistical significance was defined as $P < 0.05$.

RESULTS

ALDOSTERONE INCREASES 12- AND 15-LO-2 mRNA EXPRESSION

RT-PCR analysis of the total RNA prepared from VSMC confirmed the mRNA expression of MR in these cells (data not shown). We examined the effects of aldosterone on the expression of two lipoxygenase enzymes, which we have previously identified in cultured human VSMC and T/G HA-VSMC, that is, platelet-type 12-LO, and 15-LO type 2 [Limor et al., 2001, 2005]. Aldosterone treatment (1 nmol/L; 24 h) increased the expression of 12-LO mRNA, which was blocked by co-treatment with 100-fold excess of MR antagonists, spironolactone (100 nmol/L), and eplerelone (100 nmol/L; Fig. 1A). When quantitatively assessed, aldosterone elicited a 70% increase in 12-LO mRNA, which was totally blocked by either spironolactone or eplerelone (Fig. 1B). Similar effects of aldosterone, which were likewise inhibited by spironolactone and eplerelone were also seen in human aortic smooth muscle cells T/G HA-SMC (Fig. 1C). Further quantification with real-time PCR showed that aldosterone induced a ~30-fold increase in 12-LO mRNA expression in VSMC, which was entirely blocked by spironolactone (Fig. 1D). Under the same conditions, aldosterone also increased the expression of 15-LO type 2 (50%), an effect that was blocked by spironolactone and eplerelone (Fig. 2A–C).

ALDOSTERONE INCREASES 12–LO PROTEIN EXPRESSION

Western immunoblotting of the protein extracted from VSMC performed with a human platelet-type12-LO purified antibody (exon 4-based antibody, 1:50 dilution) detected the presence of 70 kDa protein that was up-regulated by aldosterone (1 nmol/L) treatment for 24 h (data not shown). Aldosterone had no effect on the expression of the 110 kDa platelet 12-LO protein in VSMC as determined by Western immunoblotting. However, when 12-LO protein expression levels were assessed following initial immunoprecipitation with an anti-12-LO antibody, the expression of two bands of 64 and 110 kDa was ~3- and 2-fold, respectively, higher in extracts from aldosterone-treated cells (Fig. 3A–C). This increase in 12-LO protein expression was blocked by spironolactone.

ALDOSTERONE INCREASES 12- AND 15-HETE FORMATION

As shown in Figure 4A,B aldosterone (1 nmol/L) increased the formation of the products of 12- and 15-LO. The generation of 12-HETE was increased by ~2-fold and that of 15-HETE by ~50%. These aldosterone-induced increments in eicosanoid production were inhibited by spironolactone and eplerelone (100 nmol/L).

EFFECT OF ALDOSTERONE ON LDL OXIDATION

When cultured VSMC were first pre-incubated with either aldosterone (1 nmol/L) or vehicle over 24 h, and then incubated with LDL for 18 h, LDL oxidation rate as detected by the TBARS reaction was significantly higher in aldosterone-treated VSMC (Fig. 5). Further, this effect of aldosterone could be blocked by co-treatment with excess spironolactone.

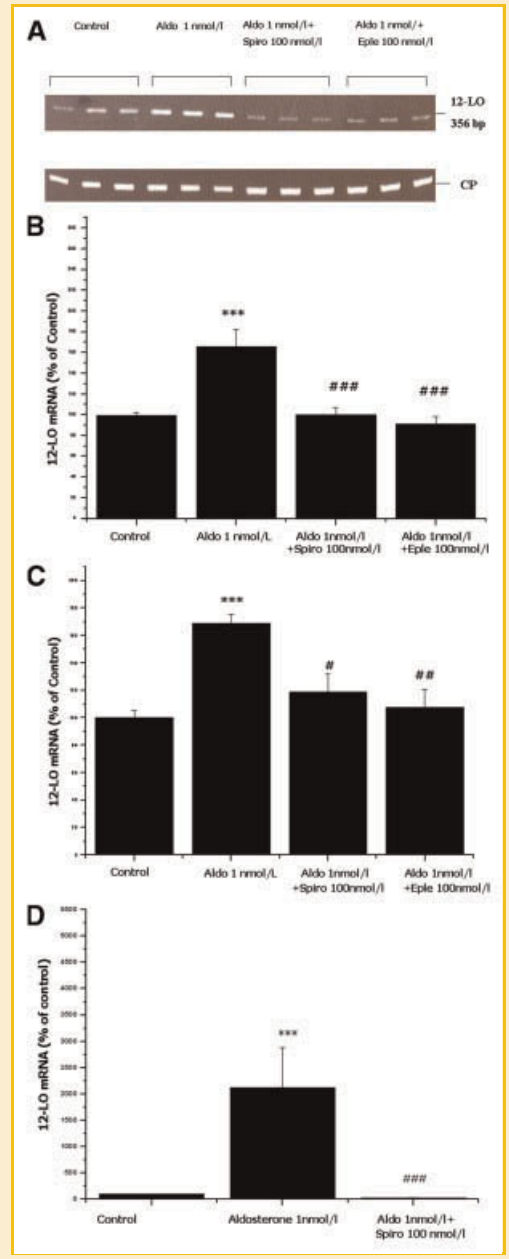

Fig. 1. A–C: Effect of aldosterone (1 nmol/L) on human platelet-type 12-LO mRNA in VSMC and T/G HA-VSMC. Total RNA was prepared from cultured VSMC after 24 h treatment in 199 medium containing 0.4% serum and then reverse transcribed and subjected to PCR with specific primers. A: Effect of aldosterone on 12-LO mRNA, in the absence or presence of either spironolactone (100 nmol/L) or eplerelone (100 nmol/L). B: Quantification of the effect of aldosterone on 12-LO mRNA in VSMC. Results are provided in arbitrary units relative to control and are the means ± SEM of 11 separate experiments; $^{***}P < 0.001$ (compared to control), $^{\#\#\#}P < 0.001$ (compared with aldosterone). Panel C shows densitometric analysis of 15-LO-2 mRNA in T/G HA-VSMC cells treated with aldosterone (1 nmol/L) in the absence or presence of either spironolactone or eplerenone (100 nmol/L). Results are expresses in arbitrary units relative to control and are the means ± SEM of eight separate experiments; $^{***}P < 0.001$(compared with control), $^{\#\#}P < 0.01$(compared to aldosterone), $^{\#}P < 0.05$ (compared with aldosterone). D: Effect of aldosterone on 12-LO mRNA, in the absence or presence of spironolactone (100 nmol/L). Data were generated by the use of real-time PCR. Results are expresses in arbitrary units relative to control and are the means ± SEM of four separate experiments.

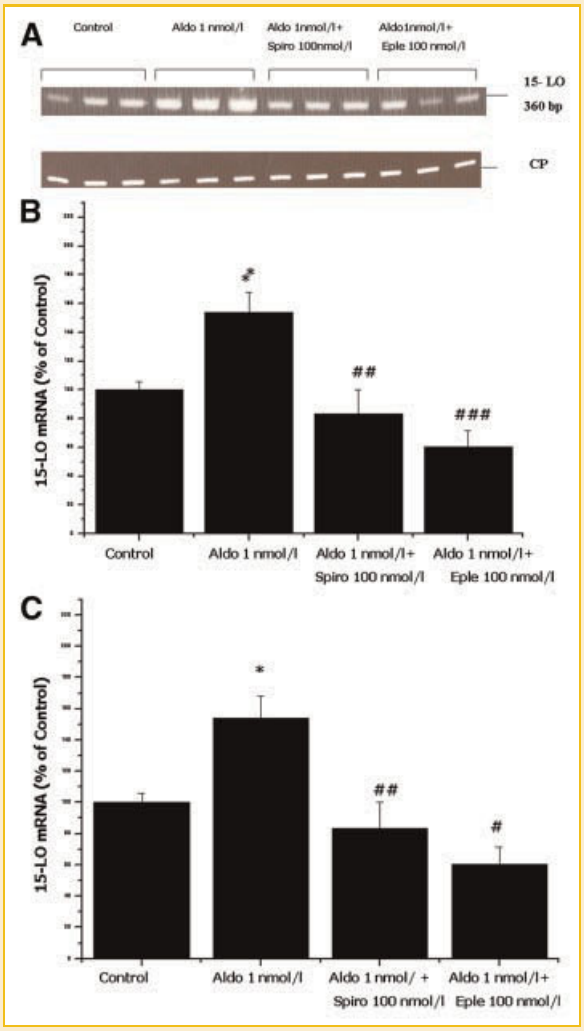

Fig. 2. A–C: Effect of aldosterone (1 nmol/L) on 15-LO-2 mRNA in VSMC and T/G HA-VSMC. Total RNA was prepared from cultured VSMC after 24 h treatment in 199 medium containing 0.4% serum. RNA was reverse transcribed and subjected to PCR with specific primers. A: Effect of aldosterone [±spironolactone (100 nmol/L) or eplerelone (100 nmol/L)] on 15-LO-2 mRNA. Panel B depicts densitometric analysis of 15-LO-2 mRNA in VSMC cell treated with either aldosterone (1 nmol/L) only or aldosterone and spironolactone (100 nmol/L), or aldosterone and eplerelone (100 nmol/L). Results are expresses in arbitrary units relative to control and are the means ± SEM of eight separate experiments; $^{*}P < 0.05$ (compared with control) $^{\#\#}P < 0.01$ (compared to aldosterone), $^{\#\#\#}P < 0.001$ (compared with aldosterone). Panel C shows densitometric analysis of 15-LO-2 mRNA in T/G HA-VSMC cells treated with either aldosterone (1 nmol/L) only or in the presence of either spironolactone (100 nmol/L) or/and eplerelone (100 nmol/L). Results are expressed in arbitrary units relative to control and are the means ± SEM of eight separate experiments; $^{*}P < 0.05$ (compared with control) $^{\#\#}P < 0.01$ (compared with aldosterone), $^{\#}P < 0.05$ (compared with aldosterone).

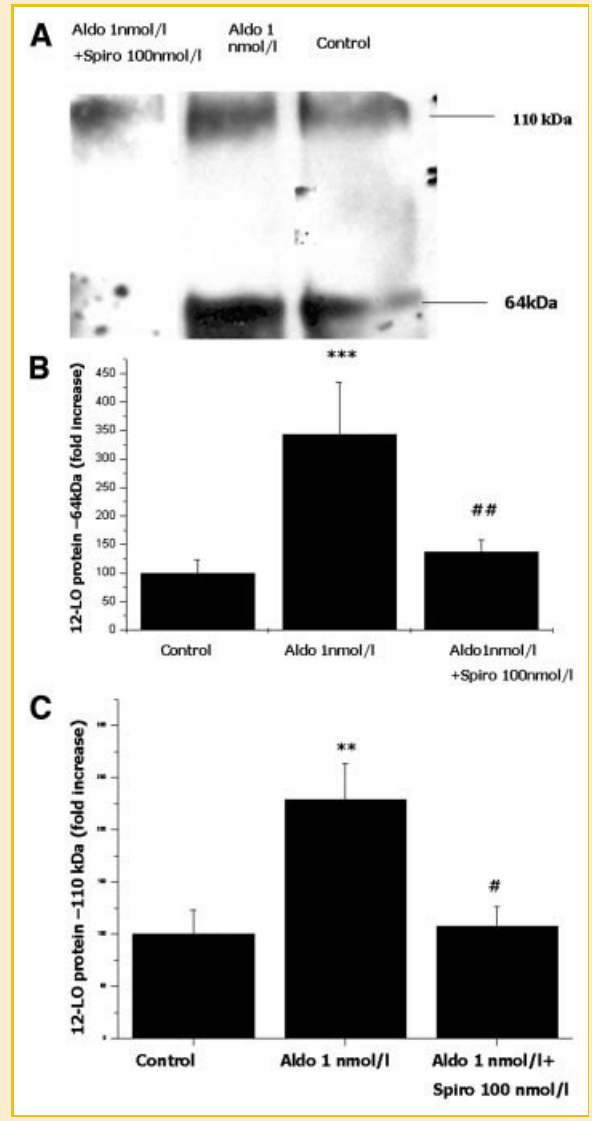

Fig. 3. Effect of aldosterone on 12-LO protein expression. VSMC were treated with aldosterone (1 nmol/L) and without and with spironolactone (100 nmol/L) for 24 h. Protein extracts prepared from the VSMC were first immunoprecipitated with a polyclonal antibody against 12-LO and, following denaturation, Western blot was performed with the same antibody. A: A representative blot of 12-LO protein expression following immunoprecipitation, showing 64 and 110 kDa bands. B: Densitometric analysis of 12-LO 64 kDa band, based on three separate experiments (means ± SEM). $^{***}P < 0.001$(compared with control) $^{\#\#}P < 0.01$ (compared to aldosterone no significantly change compared to control). C: Densitometric analysis of the 12-LO 115 kDa band, based on three separate experiments (means ± SEM). $^{**}P < 0.01$, (compared with control), $^{\#}P < 0.05$ (compared to aldosterone no significantly change compared to control.

INVOLVEMENT OF MAPK- AND EGF-RECEPTOR ACTIVATION IN ALDOSTERONE'S ACTION

Following 5 min of exposure to 1 nmol/L of aldosterone, phosphorylated ERK1/2 in cultured VSMC increased by ~2-fold, which resembled the response to 100 nmol/L of angiotensin II (Fig. 6A). The stimulatory effect of aldosterone on ERK1/2 phosphorylation was attenuated by spironolactone (100 nmol/L; 30 min pre-incubation with the antagonist; Fig. 6A). We assessed the functional role of MAPK-kinase in aldosterone-induced 12-and 15-LO expression by the use of the MAPK-kinase inhibitor UO126 (5 μmol/L; 24 h) which entirely blocked aldosterone-dependent mRNA expression of both enzymes (Fig. 7A–C). We also assessed the potential role of EGF-receptor-dependent mechanism in the effects of aldosterone: while the EGF-receptor inhibitor AG 1478 had no effect on

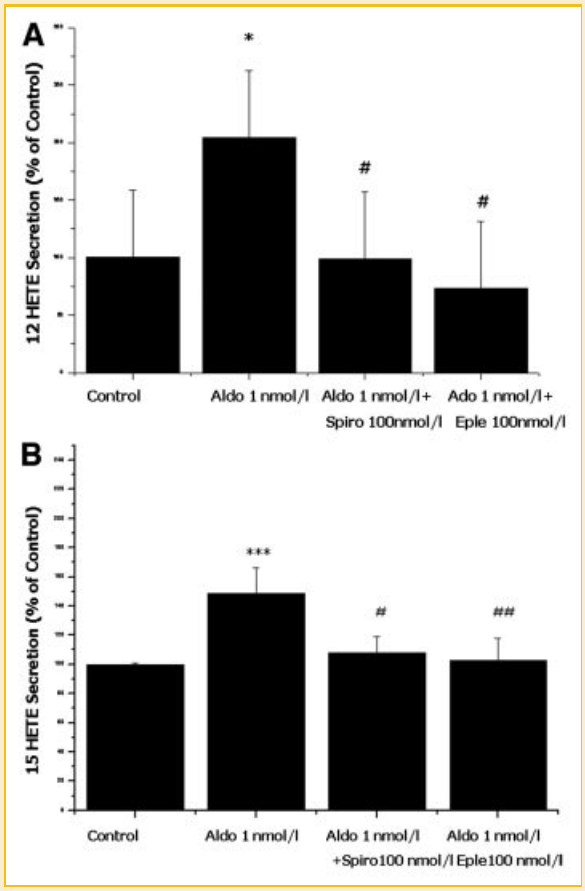

Fig. 4. A,B: Effect of aldosterone (1 nmol/L), in the absence and presence of spironolactone (100 nmol/L) or eplerelone (100 nmol/L) on the secretion of 12- and 15-HETE in cultured VSMC. The cells were pretreated with aldosterone (nmol/L) for 24 h. 12- and 15-HETE were measured after 10 min aldosterone treatment in the next day of the experiment. A: Aldosterone-induced 12-HETE secretion. Results are the means ± SEM of nine separate experiments. $^{*}P < 0.05$ (compared to control) $^{\#}P < 0.05$ (compared to aldosterone). B: Aldosterone-induced 15-HETE secretion. Results are the means ± SEM of 18 separate experiments; $^{***}P < 0.001$ (compared to control), $^{\#}P < 0.05$ (compared to aldosterone), $^{\#\#}P < 0.01$ (compared to aldosterone).

aldosterone-ERK1/2 phosphorylation (Fig. 6B), it completely blocked aldosterone-stimulated 12- and 15-mRNA expression (Fig. 7A–C). Finally, aldosterone-induced 12-LO mRNA expression could be also blocked by the Src inhibitor PPI (Fig. 7D).

DISCUSSION

In the present communication we provide evidence that in VSMC prepared from human umbilical arteries and in T/G HA-VSMC, a VSMC line derived from the human aorta, aldosterone increases the expression of two LO enzymes, human platelet-type 12- and 15-LO-2 and also elicits a rise in the formation of products of these enzymes, 12- and 15-HETE. We have previously reported that in addition to the typical platelet-type 12-LO, a variant form of platelet-type 12-LO mRNA, which includes two additional sequences consistent with introns D and E, is expressed in VSMC

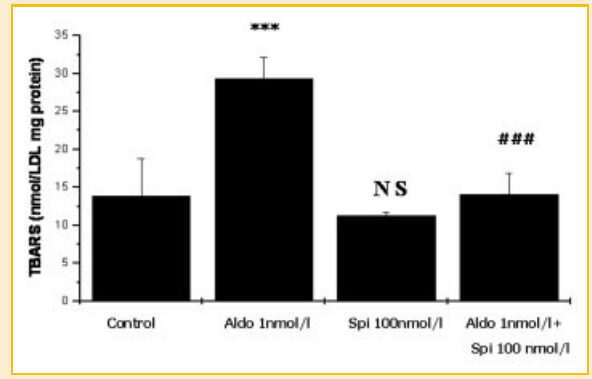

Fig. 5. Effect of aldosterone (1 nmol/L) and spironolactone (100 nmol/L) on LDL oxidation in VSMC. Cultured VSMC were treated for 24 h with aldosterone and spironolactone and LDL oxidation was measured by TBARS method after 18 additional hours during which LDL (100 μg/ml) was added. The results are the means of three separate experiments; $^{***}P < 0.001$ (compared to control); $^{\#\#\#}P < 0.001$ (compared to aldosterone).

derived from human umbilical arteries (also used in the present study) and is up-regulated by EGF [Limor et al., 2001]. In a following study we observed that this variant of the human platelet-type 12-LO was also expressed in T/G HA-VSMC, a cell line of VSMC derived from normal human aorta [Limor et al., 2005]. We now report that human umbilical artery VSMC also express 15-LO-2, which extends

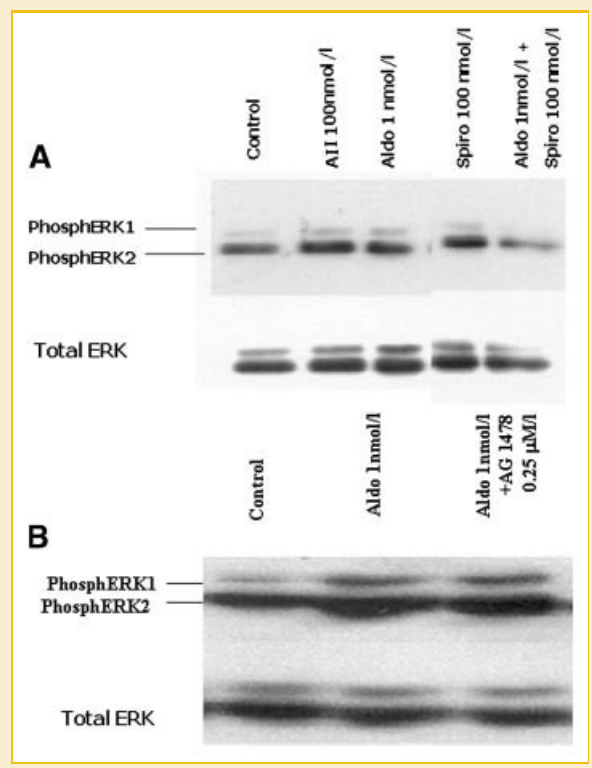

Fig. 6. Effect of aldosterone alone or aldosterone in the presence of spironolactone (A) or the EGF-receptor antagonist AG 1478 (B) on MAPK activation. As shown, aldosterone increased ERK1 and ERK2 phosphorylation, which was inhibited by spironolactone but not by AG 1478.

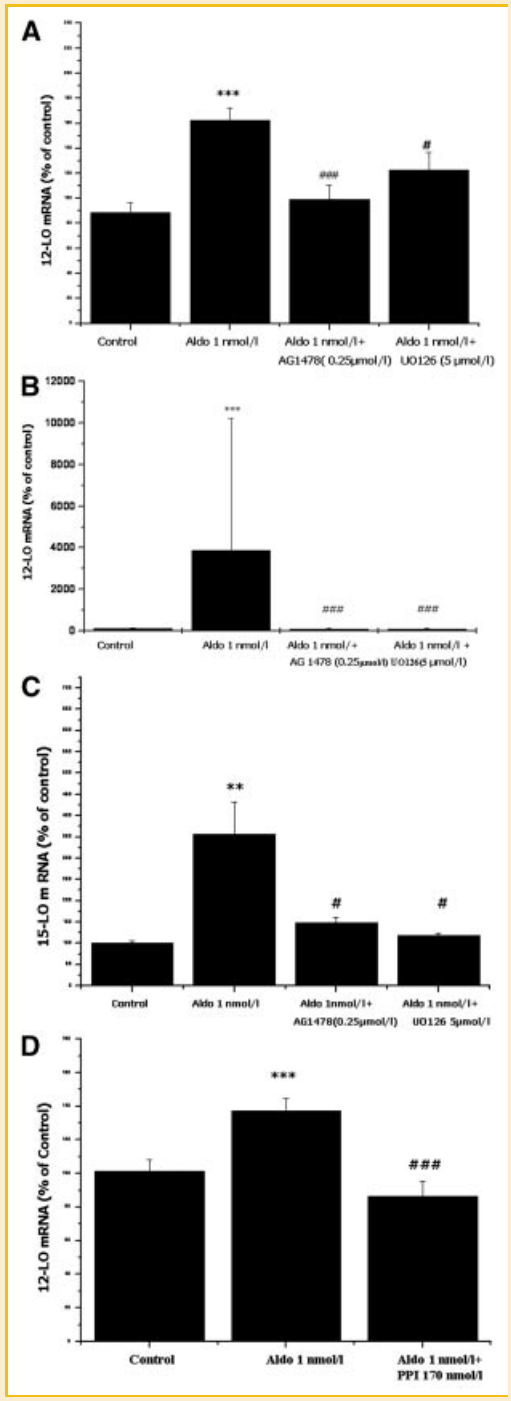

Fig. 7. A–D: Effect of the EGF-receptor inhibitor AG1478 (0.25 μmol/L), the MAPK-kinase inhibitor UO126 (5 μmol/L) and the Src inhibitor PPI on aldosterone-induced 12- and 15-LO-2 mRNA expression. The results are means of five separate experiments. A: Effects on 12-LO mRNA; $^{***}P < 0.001$(compared to control), $^{###}P < 0.001$ (compared to aldosterone); $^{#}P < 0.05$ (compared to aldosterone). B: Same effects shown in A, quantified by real-time PCR; $^{***}P < 0.001$ (compared to control), $^{###}P < 0.001$ (compared to aldosterone); (C) Effect of aldosterone in the absence and presence of AG1478 (0.25 μmol/L) or UO126 (5 μmol/L) on 15-LO-2 mRNA; $^{**}P < 0.01$ (compared with control); $^{#}P < 0.05$ (compared to aldosterone). D: Effects of Scr inhibitor PPI on aldosterone-induced 12-LO mRNA $^{***}P < 0.001$ (compared to control); $^{###}P < 0.001$ (compared to aldosterone).

our previous observation that this particular 15-LO isoform is present in T/G HA-VSMC [Limor et al., 2005].

The key finding in this report is that aldosterone up-regulates the expression of 12- and 15-LO-2, effects which are apparently mediated through MR expressed in VSMC and in T/G HA-VSMC, as they can be blocked by spironolactone and eplerenone. Our results also suggest the involvement of early, non-genomic elements in aldosterone-induced LO expression. First, aldosterone elicits a rapid rise (within 5 min of exposure) in ERK1/2 phosphorylation, which is similar in magnitude to the well-established effect of angiotensin II on MAPK in VSMC [Isenovi et al., 2004]. That this non-genomic event is critical for aldosterone-dependent 12- and 15-LO expression is suggested by the observation that the MEK (MAP kinase kinase) inhibitor UO126 can block aldosterone-induced LO expression. In agreement with these findings, Mazak et al. [2004] have recently reported that aldosterone-induced ERK 1/2 and JNK phosphorylation in VSMC within 10 min of exposure and that this response to aldosterone was equipotent to that elicited by angiotensin II. Our results are also in accord with findings in a renal epithelial cell line (MDCK) that aldosterone increases Na (+)/H (+) exchange by means of ERK1/2 activation [Gekle et al., 2002]. In contrast to the report by Rossol-Haseroth et al. [2004], however, who were unable to block aldosterone-triggered ERK1/2 phosphorylation in a collecting duct cell line (M-1) by MR antagonists, the induction of MEK activity in VSMC in our hands was clearly blocked by spironolactone, thus, suggesting involvement of classical MR in this early, apparently ''non-genomic'' event. Our results further indicate that aldosterone-induced 12- and 15-LO expression in human VSMC requires the involvement of the EGF receptor, as the EGF-receptor antagonist AG 1478 completely inhibited aldosterone-dependent LO expression. Src activation is apparently also important for aldosterone-induced 12-LO mRNA expression (Fig. 7D). There is previous support for the concept of EGF-receptor transactivation by aldosterone. In MDCK cells, aldosterone induced a rapid increase in EGF-receptor tyrosine phosphorylation, whereas inhibition of EGF-receptor kinase abolished aldosterone-induced signaling [Gekle et al., 2002]. EGF-receptor transactivation has been linked to Src activation in several systems [Grossmann and Gekle, 2007] and pharmacological inhibition of Src was specifically shown to inhibit rapid aldosterone signaling in Chinese hamster ovary (CHO) cells transfected with the human MR [Grossmann et al., 2005]. That Src inhibition abolished aldosterone-dependent 12-LO expression is consistent with the latter observation and further links early, non-genomic aldosterone-related signaling to nuclear mineralocorticoid effects such as the induction of 12-LO. In addition to the apparent rapid signaling of aldosterone via EGF-receptor-dependent mechanisms, aldosterone was shown to up-regulate EGF-receptor expression [Krug et al., 2003]. Since EGF receptor can signal through ERK1/2 in VSMC [Iwasaki et al., 2000] and given the well-established stimulatory effect of EGF on human platelet-type 12-LO [Chang, 2003] it would be tempting to speculate that aldosterone increases 12- and 15-LO expression in VSMC via transactivation of EGF receptors, followed by down stream MEK activation. However, although both MEK inhibition and EGF receptor blockade inhibited aldosterone-induced LO expression in this study, the EGF-receptor antagonist could not block

aldosterone-dependent ERK1/2 phosphorylation. This would suggest that while aldosterone-induced up-regulation of LO in VSMC requires both EGF-receptor activity and MEK activity, these signaling elements operate independently, in parallel rather than sequentially, and converge to increase LO transcription. The signaling pathway by which EGF-receptor transactivation presumed increase LO and HETES should be further investigated.

There are two important implications to the stimulatory effect of aldosterone on 12- and 15-LO in VSMC. First, exposure of VSMC to aldosterone results in increased generation of highly bioactive LO products, such as 12- and 15-HETE, whose formation is presumed to be preceded by the transient generation of intermediate precursor hydroperoxy derivatives, 12- and 15 hydroperoxyeicosatetraenoic acids (HPETE). These and other 12- and 15-LO products can affect VSMC signaling and may therefore mediate some of the presumed effects of aldosterone in the vasculature. For example, 12-HETE is involved in agonist-mediated VSMC Ca++ transients [Saito et al., 1992; Stern et al., 1993] and increases the generation rate of diacylglycerol, a product of phospholipase C [Ohta et al., 1995]. 12- and 15-HETE are required for tyrosine kinase-dependent activation (phosphorylation) of phospholipase D [Parmentier et al., 2001]. Such rapid interactions may result in augmentation of VSMC contractility [Kuhn and Chan, 1997]. 12-HETE was shown to directly increase VSMC replication and migration and 12- and 15-HETE may also exert anti-apoptotic effects [Nishio and Watanabe, 1997]. Both eicosanoids signal through the RAC1/PAK1/MEKK1/SEK1/JNK/c-JUN pathway, which promotes cell growth, and can activate p38 MAPK, probably by upstream activation of PAK1 [Wen et al., 1996, 2000]. Acting extracellularly, HETES released from VSMC may exert pro-inflammatory effects; such as presumably induced by aldosterone per se [Rocha et al., 2002]. For example, in human endothelial cells, 15-HPETE and 12-HETE as well as aldosterone were reported to induce surface expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1, endothelial-leukocyte adhesion molecule (ELAM)-1, and vascular cell adhesion molecule (VCAM)-1 [Sultana et al., 1996; Caprio et al., 2008]. Additionally, the highly reactive hydroperoxy precursors of 12- and 15-HETE, 12- and 15-HPETE may damage neighboring cells [Thollon et al., 1995], thus, providing potential further explanation for vascular inflammatory processes attributed to aldosterone.

The second potential sequel of the aldosterone-induced increase in 12- and 15-LO expression is that the capacity of VSMC for LDL oxidation might increase as well. Indeed, aldosterone-treated cells show increased LDL oxidation (Fig. 5), presumably due to the increased expression of 12- and or 15-LO. Although the LDL-oxidative effect of aldosterone was blocked by spironolactone, our results do not exclude the possibility that mechanisms other than LO enzymes were involved in aldosterone-induced LDL-oxidation. However, a large body of evidence implicates lipoxygenase enzymes in LDL oxidation in macrophages [Kuhn et al., 1997; Belkner et al., 1998; Cyrus et al., 1999; Yamashita et al., 1999; Xu et al., 2001]. Since lipoxygenase enzymes can oxidize LDL even in cell-free systems [Noguchi et al., 2002], the conjecture that aldosterone increases LDL oxidation in VSMC by increasing 12 HETE, and particularly 15-LO expression appears reasonable. Moreover, the 15-LO metabolite 15-HPETE, an obligatory transient precursor of 15-HETE generated through 15-LO, was shown an enhance the non-enzymatic oxidation of LDL particles, such that once it reaches a critical concentration within LDL particles, its pro-oxidant potency greatly exceeds even that of hydrogen peroxide [Navab et al., 2001, 2004].

In summary, aldosterone increased 12- and 15-LO expression in human VSMC, in association with increased 12- and 15-HETE generation and enhanced LDL oxidation. Thus, aldosterone may directly augment VSMC contractility, growth, and migration by increasing 12- and 15-HETE and contribute to LDL oxidation via 15-LO-dependent mechanisms. Such effects may aggravate the well-established vascular inflammatory influence of aldosterone [Rocha et al., 2002].

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