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ACTIVATION OF PROSTACYCLIN SYNTHESIS IN CULTURED AORTIC SMOOTH MUSCLE CELLS BY 'DIURETIC–ANTIHYPERTENSIVE' DRUGS

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Abstract—In cultured smooth muscle cells of rat aorta, four diuretic agents, furosemide, bumetanide, cicletanide and piretanide (all at 10^{-6}–10^{-5} M), significantly enhanced the transformation of exogenously added arachidonic acid (AA) to prostacyclin. Studies with cultured smooth muscle cells and human leukocytes revealed that these same agents failed to inhibit lipoxygenase pathways. Taken together, these results indicate that the diuretic properties of these agents might be associated with a general activation of the AA cascade.

Recently, the physiological and pathological roles of prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) have attracted much attention. Many investigations support the hypothesis than an imbalance in the synthesis of these two icosanoids may be involved in some vascular diseases [1]. Production of PGI_2 in the vascular wall was attributed originally to the intimal surface [2], but medial smooth muscle cells also produce significant quantities both *in vivo* [3, 4] and under culture conditions [5]. The capacity of the vascular wall to synthesize PGI_2 appears to be altered under different pathological conditions, including atherosclerosis [6, 7]. On the other hand, different drugs, particularly diuretics, appear to increase the release of PGI_2 from the vessel wall [8–10]. However, the mechanisms of action of these drugs on the arachidonic acid (AA) cascade have not been clarified [11–16].

In this report, using cultured aortic smooth muscle cells, we demonstrate that several diuretics (furosemide, bumetanide, piretanide) and a new antihypertensive drug (cicletanide—proposed ICD) enhanced the production of PGI_2 from exogenously added AA, and that this effect is not mediated through an inhibition of lipoxygenase pathways. The ineffectiveness of these drugs on lipoxygenase activities was further confirmed using human leukocytes.

MATERIALS AND METHODS

Materials. Cicletanide (1,3-dihydro-3-(4′-chlorophenyl)-7-hydroxy-6-methyl-furo [3, 4-c] pyridine; [17]) was produced by IBH Research Laboratories (Paris, France); furosemide and piretanide were kindly supplied by Hoechst (Paris, France); bumetanide was a gift of Leo Pharmaceutical (Paris, France); Prostaglandins were a gift of Dr. J. Pike (Upjohn, Kalamazoo, Mich.); leukotrienes were kindly supplied by J. Rokach (Merck Frosst, Montreal, Canada).

Arachidonic acid was purchased from Sigma Chemical Co. (St Louis, MO) and [1-^{14}C] AA was from the Radiochemical Center Amersham, and these substances were always purified on silicic acid columns before use. Hydroxyeicosatetraenoic acids, namely 15-, 12- and 5-HETEs, were prepared and purified by high performance liquid chromatography [18].

Aortic smooth muscle cell preparation. Aortic smooth muscle cells were obtained from explants of thoracic aorta from adult male rats essentially as described by Ross [19]. Cells were grown at 37° in 25 cm^2 plastic flasks in an atmosphere of 5% CO_2 in air using HAM F10 growth medium supplemented with 20% fetal calf serum during the first few weeks. When confluency was achieved, cells were trypsinized and subcultivated in a 1:3 split ratio in 10% fetal calf serum supplemented HAM F10 medium. All experiments were done using cells below passage number 8.

Metabolism of [1-^{14}C] *arachidonate by cell homogenates.* Cells were scraped with a rubber policeman, suspended in 2 ml of 0.05 M Tris–HCl, 0.15 M NaCl (pH 7.4) buffer and homogenized. (Sonication was avoided in order to minimize free radical formation.) Aliquots (0.2 ml) of resultant suspensions were used for protein determination according to the method of Bradford [20]. The reaction mixture had the following composition: cell homogenate, 1 mg of protein; [1-^{14}C] AA (50 Ci/mole), 10 nmoles in 10 μl absolute ethanol. The final reaction volume was 1 ml.

Drugs (10–100 μM) were added to the incubation mixture 10 min prior to adding AA. Incubations were carried out at 37° for 20 min in the dark with shaking, and stopped either by acidification to pH 3.0 with citric acid, or by addition of 1.5 volume of methanol. Extractions were performed as previously described [21]. For thin layer chromatography, the extracts were dissolved in ethyl acetate, and the plates were developed using the upper phase

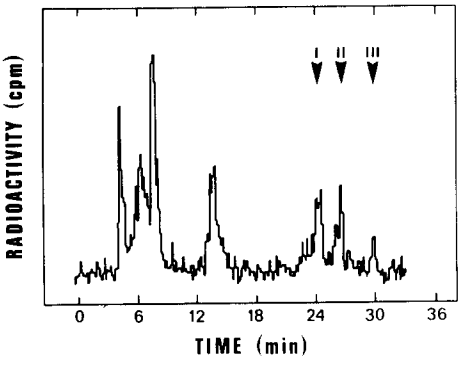

Fig. 1. Typical HPLC profile obtained from cultured rat aortic smooth muscle cells on C_{18} reverse phase column. Isocratic elution MeOH:H_2O (75:25) AcOH 0.02% (pH 5.7); flow rate 1.5 ml/min; arrow-head defines the position of unlabelled standards. I, 15-HETE; II, 12-HETE; III, 5-HETE.

of ethyl acetate/iso-octane, acetic acid/water (110:50:20:100, v/v). Standards were visualized by iodine vapors, and the radioactive products were located by autoradiography using Kodak X-ray films (X-Omat) after two days exposure. Radioactive zones were scraped from the plates and counted. For high performance liquid chromatography, ether extracts were evaporated, dissolved in methanol, and treated at 4° in darkness with diazomethane in ether to form the methyl esters. After evaporation, dry residues were dissolved in absolute methanol and injected onto a μ Bondapack C_{18} analytical column (Waters Associates). The mobile phase was composed of 75% methanol, 25% water and 0.02% acetic acid (pH adjusted to 5.7 with NH_4OH). Isocratic elution was performed at a flow rate of 1.5 ml/min. Column effluent was continuously monitored for u.v. absorbance at 232 nm, and 1.5 ml fractions were collected. Portions of each 1.5 ml fraction were monitored for radioactivity by scintillation counting. In some experiments, the column effluent was directly monitored for counting in a Flow One (Kontron) radiometer using Lumaflow II as liquid scintillator (Fig. 1).

Determination of lipoxygenase inhibition in human leukocytes plus platelets. Suspensions of human leukocytes plus platelets were prepared from citrated blood as follows: immediately after collection, the blood was centrifuged (200 g × 15 min, 20°) and the platelet-rich plasma was removed; leukocytes were separated from the red blood cell pellets after treatments with dextran and ammonium chloride as previously described [22] (Ficoll-paque separation of mononuclear and polymorphonuclear leukocytes was not performed). The leukocytes were finally resuspended in Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) at the concentration of 10–15 × 10^6 cells/ml for incubation; the platelet/leukocyte ratio was ≤ 3.0.

Compounds to be tested were pipetted into polypropylene test tubes (18 μl of ethanolic solutions followed by 2 ml of the cell suspension which was then preincubated for 20 min at 37°). Calcium and magnesium chlorides were added to the tubes at the beginning of this pre-incubation period at final concentrations of 2 and 0.5 mM, respectively; the cells were then stimulated with the ionophore A 23187 (2 μM, final concentration); after 5 min of incubation at 37°, the reaction was stopped by addition of 2 ml of methanol. The concentrations of 5-lipoxygenase products were measured by reversed-phase, high performance liquid chromatography (RP-HPLC) using the procedure reported previously [22] with minor modifications [23].

RESULTS

Drug effects on AA transformation in cultured aortic smooth muscle cells

In the first series of experiments, the metabolism of exogenous [1-^{14}C] AA by cultured smooth muscle cells was studied after 10 min pre-incubation of the cells with the drugs at 10^{-5} M. Under these conditions, the four drugs tested produced a significant enhancement of the oxidative transformation of the substrate, essentially by stimulation of the cyclooxygenase pathway (Table 1). Furthermore, a significant enhancement of PGI_2 formation, measured as 6-oxo-$PGF_{1\alpha}$, was observed when data were expressed as percent of transformed AA (Table 2).

Table 1. Effects of drugs on the metabolism of exogenous [^{14}C] AA by cultured rat aortic smooth muscle cells

Drug	Total transformed	C.O. pathway	L.O. pathway	C.O./L.O.
Control (no drug)	17.5 ± 3.6	12.5 ± 3.4	5.0 ± 0.8	1.8
Furosemide	41.0 ± 8.5	32.3 ± 7.6	6.5 ± 1.2	3.9
Cicletanide	40.0 ± 7.3	32.0 ± 10.1	6.2 ± 1.4	4.3
Bumetanide	48.3 ± 16.2	35.6 ± 12.7	10.5 ± 3.7	2.7
Piretanide	40.6 ± 11.4	30.5 ± 10.3	8.4 ± 2.1	2.7

Cells were pre-incubated 10 min with the drugs (10^{-5} M) and were incubated for 20 min with [^{14}C] AA and analysed by TLC as described in Materials and Methods. Results are expressed as percent of the total radioactivity recovered from the incubation medium (means ± S.E.M. of three experiments with the same cell strain). C.O. indicates the summation of 6-keto-$PGF_{1\alpha}$ (R_f = 0.11), $PGF_{2\alpha}$ (R_f = 0.25), PGE_2 (R_f = 0.36) and HHT (R_f = 0.74); L.O. indicates the spot on TLC chromatograms that contained lipoxygenase products, mainly 12-HETE and 15-HETE (R_f = 0.77). All drugs produced significant increases in total [^{14}C] AA transformed and in the C.O. pathway ($P < 0.05$ or $P < 0.01$; Student's t-test; two-tailed), but had no effect on the L.O. pathway ($P > 0.1$).

Table 2. Effects of drugs on prostanoid formation by cultured rat aortic smooth muscle cells

Drug	6-Keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2
Control (no drug)	9 ± 2.3	1 ± 1.2	1 ± 0.9
Furosemide	25 ± 6.8	3 ± 2.2	3 ± 1.3
Cicletanide	25 ± 8.4	2 ± 1.4	3 ± 0.6
Bumetanide	23 ± 12.3	5 ± 3.6	4 ± 1.5
Piretanide	22 ± 4.7	3 ± 2.1	4 ± 0.9

Cells were incubated and results are expressed as described in Table 1. Note that cells were used at passage No. 3.

The effect of cicletanide was further explored by incubating cells with increasing concentrations of drugs (10^{-5} to 10^{-3} M). As shown in Fig. 2, the enhancement of PGI_2 formation was dose-dependent. In contrast, the drug did not affect the lipoxygenase pathway at all concentrations studied.

The lipoxygenase was further studied using HPLC analyses after treatment of the cells with cicletanide (10^{-4} M and 10^{-5} M) and furosemide (10^{-5} M). In these studies, we confirmed the activation of the oxidative metabolism of AA. As shown in Fig. 1, three main monohydroxy derivatives were isolated from the incubation medium of rat aortic smooth muscle cells by HPLC (15-HETE, 12-HETE and 5-HETE). Figure 3 shows that drug treatment did not significantly modify this typical profile.

Effects of diuretics on the lipoxygenase pathways of human leukocytes and platelets

As shown in Figs. 4, 5, 6 and 7 the drugs (10^{-7} M to 10^{-4} M) did not induce any significant change in the synthesis of lipoxygenase products.

DISCUSSION

The results presented herein show that four diuretic agents induced a significant enhancement of the transformation of AA through the cyclooxygenase pathway, while not affecting the lipoxygenase pattern of arterial smooth muscle cells. It is noteworthy that a significant increase in PGI_2 formation was obtained with relatively low concentrations (10^{-5} M) of cicletanide and furosemide. In addition, the drugs tested did not alter the lipoxygenase pathways in human leukocytes and platelets.

Our results do not support any of the mechanisms previously proposed for explaining the increased levels of vasodilatator prostaglandins induced by diuretics and related drugs; i.e. stimulation of AA release [11], or inhibition of the catabolic enzymes (15-OH prostaglandin dehydrogenase and 9-oxo-reductase) [12, 13]. Indeed, since experiments were performed using exogenous AA, any modification of the transformation probably does not involve

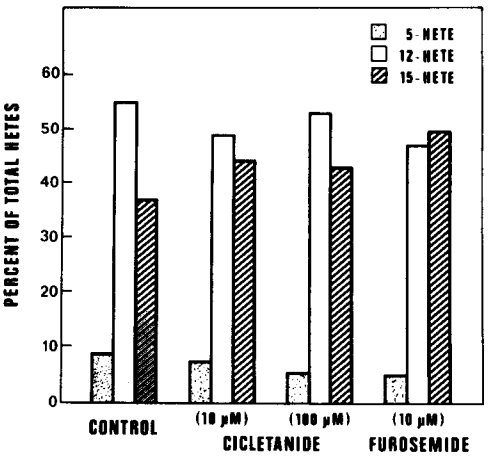

Fig. 3. Effects of cicletanide and furosemide on the monohydroxylated compounds synthesized by arterial smooth muscle cells.

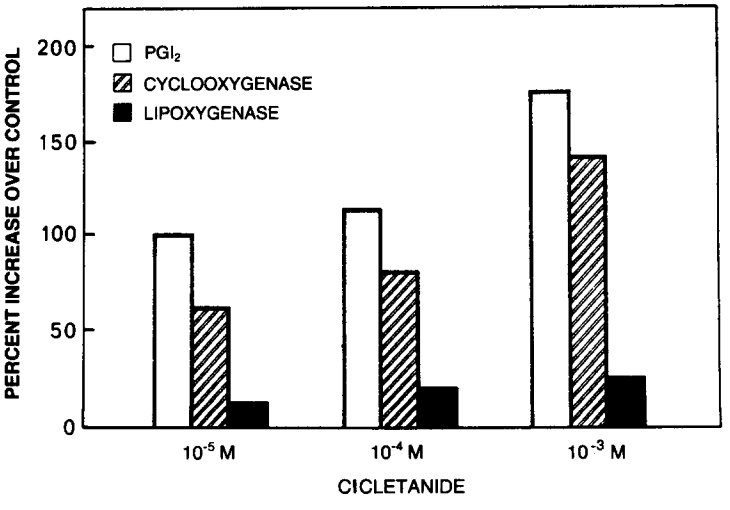

Fig. 2. Concentration–response relationship of the effects of cicletanide on cyclooxygenase and lipoxygenase activities and PGI_2 production. The lack of correspondence between values shown here and those provided in Table 2 is due to a difference in the number of passages of the cells. In this figure cells were used at passage No. 7.

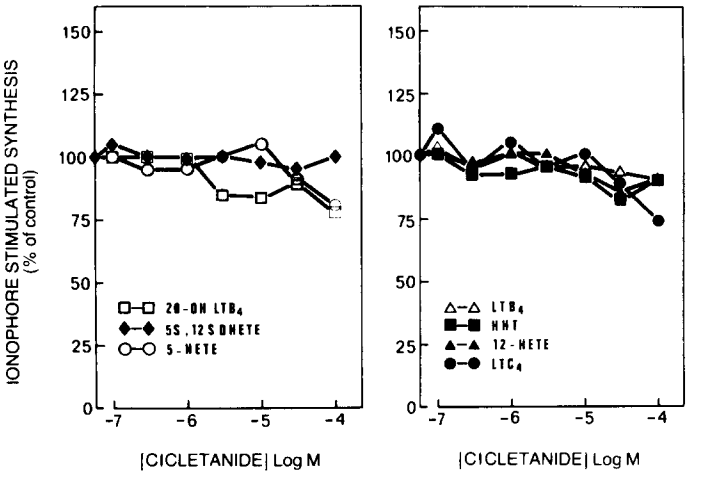

Fig. 4. Effects of cicletanide on human leukocyte plus platelet lipoxygenase pathways (mean values; N = 3).

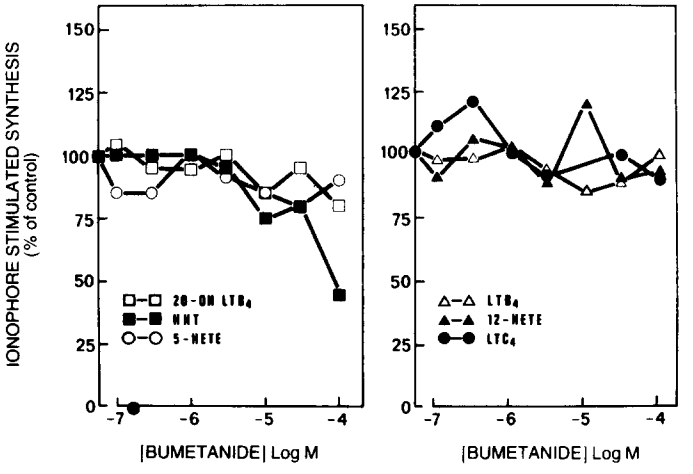

Fig. 5. Effects of bumetanide on human leukocyte plus platelet lipoxygenase pathways (mean values; N = 3).

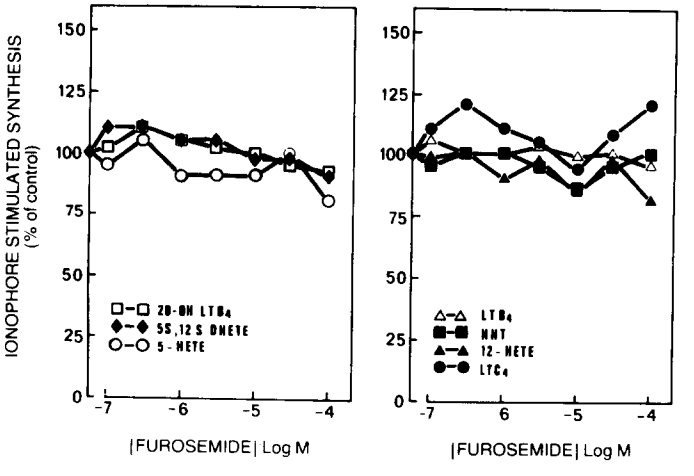

Fig. 6. Effects of furosemide on human leukocyte plus platelet lipoxygenase pathways (mean values; N = 3).

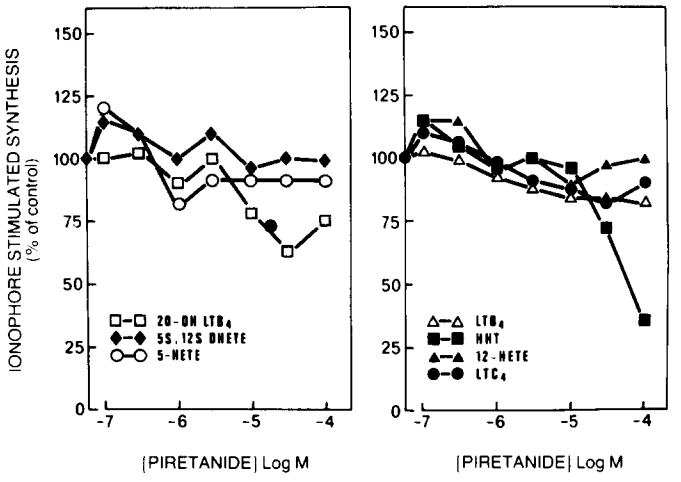

Fig. 7. Effects of piretanide on human leukocyte plus platelet lipoxygenase pathways (mean values; N = 3).

changes in acyl-hydrolase activity. However, under *in vivo* conditions involving the transformation of endogenous AA, a possible inhibitory action of these drugs on acyl-hydrolase activity cannot be excluded since recent results have indicated that inhibition of endogenous acyl-hydrolase activities actually leads to a stimulation of the transformation of exogenously-added [^{14}C]AA [24]. On the other hand, under our experimental conditions, formation of the hydrolysis product of PGI_2, 6-oxo-$PGF_{1\alpha}$, remained unchanged when expressed as % of the total cyclooxygenase products, as compared to control experiments.

The results also demonstrate an imbalance between cyclooxygenase and lipoxygenase activities. Therefore, we conclude that diuretics and the related drug used (cicletanide), produced a true activation at the level of cyclooxygenase. The exact mechanism of this activation remains to be defined. The stimulatory action of diuretics on cyclooxygenase may contribute to their antihypertensive effect through an enhancement of PGI_2 production.

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