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Furosemide Enhances the Release of Endothelial Kinins, Nitric Oxide and Prostacyclin

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ABSTRACT

Despite a wealth of data, the mechanism of the direct dilator effect of furosemide on the systemic arterial and venous systems is far from being satisfactorily understood. Therefore, we investigated whether furosemide is capable of stimulating the production of the endogenous vasodilators nitric oxide and prostacyclin in primary cultured bovine aortic endothelial cells by an enhanced synthesis and release of endothelium-derived kinins. Nitric oxide production was assessed in terms of intracellular guanosine cyclic-3′,5′ monophosphate accumulation; kinin and prostacyclin release were determined by specific radioimmunoassays. Furosemide concentration- and time-dependently increased the formation of nitric oxide and prostacylin. Maximal increases of both autacoids were already obtained after a 5-min incubation with 3×10^{-7} to 10^{-6} mol/l of furosemide. In the same concentration range, furosemide led to an enhanced release of kinins into the supernatant of the cells. This observation was supported by the inhibitory effect of the specific B_2 kinin receptor antagonist icatibant (Hoe 140) on the furosemide-induced increase of nitric oxide and prostacyclin. Thus the hemodynamic effects, and in particular the direct early dilator effect, of furosemide may be explained in part by an enhanced endothelial synthesis and release of bradykinin and related kinins, which in turn stimulates endothelial autacoid formation *via* B_2 kinin receptor activation.

Earlier investigations in patients suggest that the acute systemic hemodynamic effects of furosemide, which appear within a few minutes after intravenous administration before the diuretic response occurs, are due to a direct dilator action on blood vessels and are independent of its diuretic properties (Biamino *et al.*, 1974, 1975; Dikshit *et al.*, 1973; Schenk *et al.*, 1975). Nevertheless, it has been shown that the kidneys play a role in mediating this effect, because the early dilator effect of furosemide is abolished by nephrectomy in hypervolemic anuric dogs (Bourland *et al.*, 1977) and anephric patients (Johnston *et al.*, 1983). Furthermore, rats given furosemide after bilateral uretal ligation showed a decrease in arterial blood pressure. In comparison, bilateral ligation of renal blood vessels suppressed this effect, which led to the conclusion that the blood pressure-reducing action of furosemide requires the integrity of renal vessels (Sechi *et al.*, 1990). On the basis of the latter studies, prostaglandins of renal and/or extrarenal origin appear to be involved in the early vasodilator effect of furosemide, because pretreatment with indomethacin abolished this effect. Sulindac, which predominantly affects the extrarenal synthesis of prostaglandins (Wong *et al.*, 1986), did not reverse the antihypertensive effect of diuretics in patients (Puddey *et al.*, 1985); this underscores the role of renal prostaglandin synthesis in mediating the peripheral vascular effects of furosemide. However, after furosemide treatment, plasma concentrations of arachidonic acid (Gerber and Nies 1981) or metabolites of prostacyclin (Mackay *et al.*, 1984; Johnston *et al.*, 1983) were too low to produce physiological effects (Steer *et al.*, 1980). This agrees with results from Gerkens *et al.*, (1988) indicating that renal prostaglandins are involved in the furosemide-induced release of an unidentified nonprostanoid hormone from the kidney, which produces an endothelium-dependent inhibition of sympathetic vasoconstriction in the *in situ* blood-perfused tail artery of the rat.

Few experimental data are available that show a direct dilatory action of furosemide on isolated intact blood vessels. In the isolated perfused canine lung lobe, furosemide significantly decreased the mean pulmonary arterial pressure. This effect was mimicked by prostacyclin and inhibited by indomethacin, which suggests local formation of prostacyclin

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ABBREVIATIONS: BAEC, bovine aortic endothelial cells; cyclic GMP, guanosine cyclic-3′,5′ monophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; L-NNA, N^G-nitro-L-arginine; PGI_2, prostaglandin I_2 (prostacyclin); SOD, superoxide dismutase.

in the presence of furosemide (Lundergran *et al.*, 1988). In the isolated rabbit ear artery segments, furosemide caused an inhibition of contraction induced by electric field stimulation, which is endothelium-independent (Tian *et al.*, 1991).

There is no report of a direct effect of furosemide on the endothelium, which is known to synthesize and release potent vasodilators such as acetycholine, ATP, substance P and kinins (Milner *et al.*, 1990; Wiemer *et al.*, 1994). Kinins, predominantly bradykinin, act as vasodilators in an autocrine manner by stimulating endothelial kinin receptors with subsequent synthesis and release of nitric oxide and prostacyclin (Schini *et al.*, 1990; Wiemer *et al.*, 1991). Therefore, we investigated whether cultured endothelial cells from bovine aorta respond to furosemide with an enhanced synthesis and release of kinins, nitric oxide and prostacyclin.

Materials and Methods

Endothelial cell culture. BAEC were isolated by digestion with dispase and were cultured as previously described (Wiemer and Wirth, 1992). Cells were seeded on six-well plates (Nunc Intermed, Wiesbaden, Germany), precoated with collagen R) and grown to confluence. The culture medium used for BAEC was Dulbecco's modified Eagle's/Ham's F-12 medium (1:1) containing heat-inactivated fetal calf serum (20%), penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (1 mmol/l), glutathion and L(+) ascorbic acid (each 5 μg/ml; Biotect protection medium). The purity of primary cultured BAEC was characterized by uptake of fluorescent-labeled low-density lipoprotein (Voyta *et al.*, 1984) and by negative staining for α-smooth muscle actin (Absher *et al.*, 1989).

Measurement of 6-ketoprostaglandin $F_{1\alpha}$, kinins and cyclic GMP. Primary cultures of BAEC grown to confluence in six-well plates (~200–250 μg protein per well) were used. After removal of the culture medium by aspiration, the monolayers were washed twice with 2 ml of warm (37°C) HEPES-Tyrode's solution, pH 7.4 (in mmol/l: KCL 2.7, NaCl 137, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.36, glucose 5, HEPES 10). Thereafter, the cells were preincubated for 15 min at 37°C with 1 ml HEPES-Tyrode's solution containing IBMX (10^{-4} mol/l). Then the drugs or solvents and SOD (3×10^{-7} mol/l) were added at the concentrations and times indicated in the results. At the appropriate time, the supernatants (incubation medium) were quickly removed and assayed for their contents of 6-ketoprostaglandin $F_{1\alpha}$(6-keto $PGF_{1\alpha}$), the stable breakdown product of prostacyclin (PGI_2), by a specific radioimmunoassay (Amersham Buchler, Braunschweig, Germany). Alternatively, the supernatants (each 1 ml) were directly transferred into EDTA solution (final concentration 10^{-4} mol/l) for determination of kinins by a radioimmunoassay (Fink *et al.*, 1985). The antibody (from Shimamoto) used in this assay did not distinguish among the three mammalian kinins bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin (almost 100% cross-reactivity relative to bradykinin). Other kinin fragments such as Des-Arg^9-bradykinin, as well as bovine low-molecular-weight kininogen, showed no significant cross-reactivity (<0.1%–0.02% relative to bradykinin) (Bönner *et al.*, 1987; Shimamoto *et al.*, 1978). For determination of intracellular cyclic GMP, the cells were immediately extracted with 0.8 ml of 1 N formic acid/acetone (v/v, 15:85) and scraped off with a rubber scraper. The cell suspensions were then sonicated for 10 sec before being centrifuged for 10 min at 1000 × *g*. The supernatants were lyophilized and resuspended in 0.25 ml sodium acetate buffer (0.05 mol/l, pH 6.2) for determination of cyclic GMP by a specific radioimmunoassay (DuPont NEN, Bad Homburg, Germany). The protein contents of the samples were measured according to the method of Lowry *et al.*, (1951). Cyclic GMP content was expressed as picomoles per milligram protein, that of 6-keto $PGF_{1\alpha}$ as nanograms per milligram protein and that of kinins as picomoles per milliliter supernatant.

The values were expressed as the mean ± S.E.M. Statistical comparisons were made using Dunnett's test. Values of $P < .05$ were considered statistically significant.

Drugs. SOD (bovine erythrocytes; specific activity, 3300 U/mg) and collagen R were purchased from Serva (Heidelberg, Germany) and DiI-Ac-LDL from Paesel + Lorei (Frankfurt/Main, Germany). Furosemide sodium salt and icatibant (Hoe 140) were obtained from our department of pharmasynthesis. All other compounds were purchased from Sigma Chemie (Deisenhofen, Germany).

Results

Effect of furosemide on cyclic GMP and kinin production in BAEC. In primary cultured BAEC, furosemide induced time- and concentration-dependent increases in intracellular cyclic GMP content. Maximal stimulation of cyclic GMP synthesis was reached by about 5 min and remained stable for at least 30 min of continuous exposure of the cells to furosemide (3×10^{-7} and 10^{-5} mol/l) (fig. 1, A and B). Preincubation (5 min) of the cells with the stereospecific inhibitor of nitric oxide synthase L-NNA (10^{-5} mol/l) totally suppressed the effect of furosemide without affecting unstimulated cells (fig. 1 B). An inhibition of the furosemide-induced cyclic GMP formation was also observed in monolayers that were preincubated (5 min) with 10^{-7} mol/l of the specific B_2 kinin receptor antagonist icatibant (Hock *et al.*, 1991) whereas basal cyclic GMP content was not influenced by icatibant (fig. 1 B). The concentration-response relation of

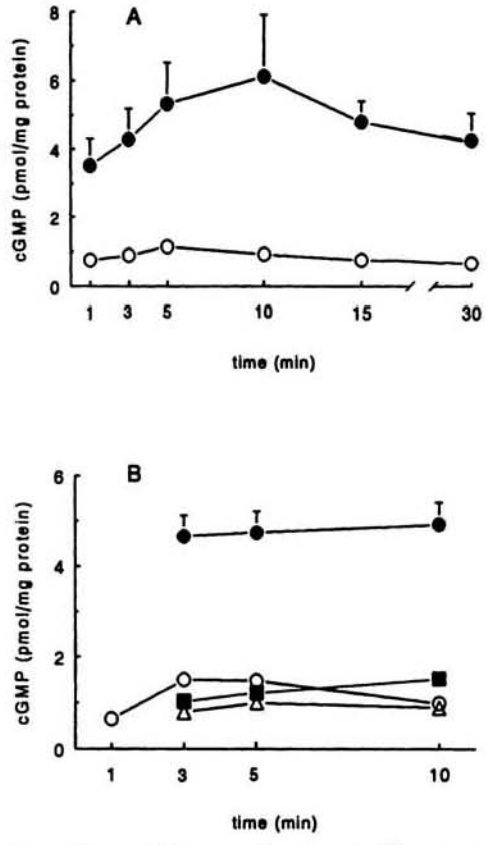

Fig. 1. A) Basal (○)- and furosemide (3×10^{-7} mol/l)-induced (●) accumulation of cGMP in primary cultured BAEC as a function of time. B) Icatibant (10^{-7} mol/l; ■) and L-NNA (10^{-5} mol/l; △) were added 5 min before the addition of furosemide (10^{-5} mol/l). Each value represents the mean ± S.E.M. of six dishes performed on two different cell batches.

furosemide is shown in figure 2. After a 10-min incubation, maximal increases in cyclic GMP were obtained by about 3×10^{-7} to 10^{-6} mol/l of furosemide with a threshold concentration of about 3×10^{-9} mol/l. Preincubation of the cells with either L-NNA (10^{-5} mol/l) or icatibant (10^{-7} mol/l) totally prevented the furosemide-induced increases in cyclic GMP. In comparison, exogenously added bradykinin (which we always used as a "standard" for the respective cell batches) led, at an optimal concentration of 10^{-8} mol/l after 1 min incubation (Wiemer *et al.*, 1991), to a little lower content of cyclic GMP (3.70 ± 0.61 pmol/mg protein *vs.* control incubation 0.61 ± 0.12 pmol/mg protein; $n = 11$) than did the optimal effective concentrations of furosemide (3×10^{-7}–10^{-6} mol/l).

In parallel to the concentration-dependent increase in intracellular cyclic GMP by furosemide, the compound enhanced the concentration of kinins in the assay media (fig. 3). Already at 3×10^{-8} mol/l of furosemide (after a 10-min incubation), an approximately 3.5-fold higher concentration of kinins (~60 pmol/ml supernatant) *vs.* control incubation was obtained. Cyclic GMP production occurring at this concentration of kinins in the assay media is approximately comparable with changes in cyclic GMP and PGI_2 induced by bradykinin (10^{-8} mol/l).

Effect of furosemide on prostacyclin (PGI_2) synthesis in BAEC. The time course of the furosemide (3×10^{-7} mol/l)-stimulated release of PGI_2 is depicted in figure 4. A plateau that remained stable for at least 30 min was reached after a 5-min incubation. The concentration-response relation of furosemide revealed maximal increases in PGI_2 release at about 3×10^{-7} mol/l, with a threshold concentration of about 3×10^{-9} mol/l (fig. 5). Preincubation with icatibant (10^{-7} mol/l) significantly inhibited this effect without affecting the basal release of PGI_2. Thus the time- and concentration-dependent effect of furosemide on PGI_2 release was quite parallel to that observed for cyclic GMP accumulation (see figs. 1 and 2). In comparison, bradykinin at a concentration of 10^{-8} mol/l (1-min incubation) caused the release of an approximately similar amount of PGI_2 (752 ± 70 ng/mg protein *vs.* control incubation 292 ± 30 ng/mg protein; $n = 7$).

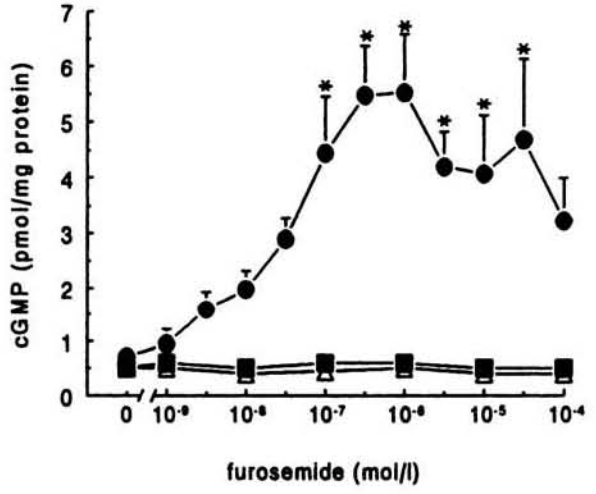

Fig. 2. Effect of furosemide (●) on the accumulation of intracellular cGMP in primary cultured BAEC as a function of concentration (10-min incubation). Icatibant (10^{-7} mol/l; ■) and L-NNA (10^{-5} mol/l; △) were added 5 min before the addition of the respective concentrations of furosemide. Results are expressed as the mean ± S.E.M. Experiments (n = 7–11, performed on six different cell batches) were done on triplicated dishes. * $P < .05$ *vs.* control (abscissa zero point).

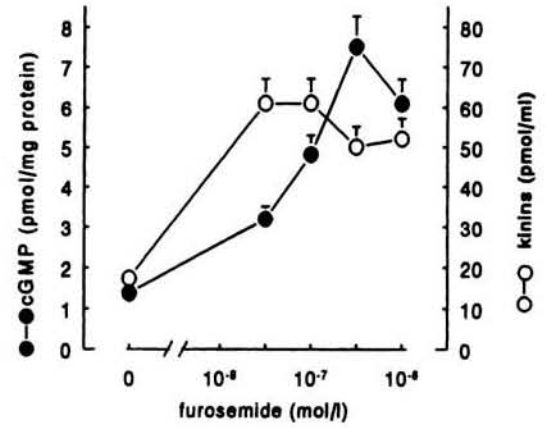

Fig. 3. Relationship between the accumulation of intracellular cGMP (left ordinate) and that of kinins (right ordinate) in the supernatant of primary cultured BAEC incubated with furosemide (10-min incubation). cGMP and kinin concentrations were determined concomitantly in the same dish. Each value represents the mean ± S.E.M. of six dishes performed on two different cell batches.

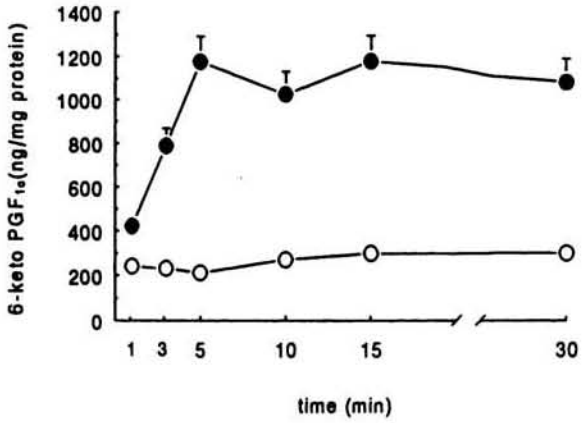

Fig. 4. Basal (○) and furosemide (3×10^{-7} mol/l)-induced (●) accumulation of 6-keto $PGF_{1\alpha}$ in the supernatant of primary cultured BAEC as a function of time. Each value represents the mean ± S.E.M. of six dishes performed on two different cell batches.

Discussion

This study has shown that furosemide stimulates the formation of cyclic GMP and PGI_2 in primary cultured BAEC. Similar findings to those with BAEC were obtained with primary cultured endothelial cells from cardiac microvessels of the rat (unpublished data). An enhanced synthesis of endothelial cyclic GMP had been well documented as an index of endothelial nitric oxide synthesis and release in response to bradykinin, ATP, ADP and calcium ionophore (Boulanger *et al.*, 1990; Martin *et al.*, 1988; Schini *et al.*, 1990). In these investigations it was shown that the stimulated increase in endothelial cyclic GMP was inhibited by methylene blue, an inhibitor of the activation of soluble guanylyl cyclase and by hemoglobin, a scavenger of nitric oxide. Increases in endothelial cyclic GMP generation by stimulation of a soluble guanylyl cyclase through nitric oxide were also demonstrated by the inhibitory effect of the stereospecific inhibitor of nitric oxide synthase L-NNA (Mülsch and Busse 1990) on bradyki-

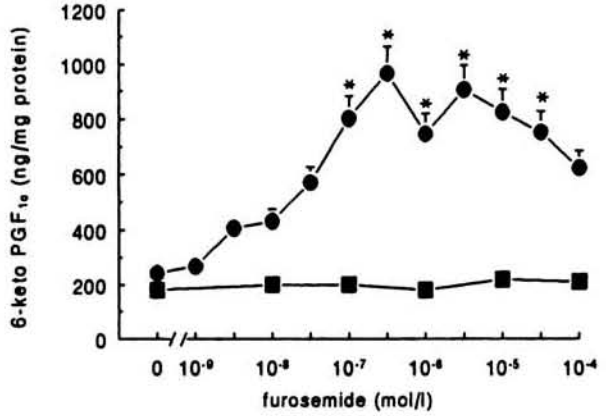

Fig. 5. Effect of furosemide (●) on the accumulation of 6-keto $PGF_{1\alpha}$ in the supernatant of primary cultured BAEC as a function of concentration (10-min incubation). Icatibant (10^{-7} mol/l; ■) was added 5 min before the addition of the respective concentrations of furosemide. Results are expressed as the mean ± S.E.M. Experiments (n = 7–11, performed on six different cell batches) were done on triplicated dishes. * $P < .05$ *vs.* control (abscissa zero point).

nin-induced cyclic GMP generation (Dubbin *et al.*, 1990; Hecker *et al.*, 1993; Wiemer *et al.*, 1991; Wiemer and Wirth, 1992). Thus the observed prevention of the furosemide-induced increase in endothelial cyclic GMP indicates an enhanced generation of nitric oxide by this compound.

The increase in cyclic GMP by furosemide is associated with an augmented release of endothelial kinins. This is supported by the observation that preincubation of the cells with the specific B_2 kinin receptor antagonist icatibant abolished the furosemide-induced increase in nitric oxide synthesis as well as the release of PGI_2. It also indicates the contribution of endogenous bradykinin in this response. The slight dissociation of the concentration-response relation of furosemide on cyclic GMP and kinins (fig. 3) cannot be satisfactorily explained. It may be that furosemide at higher concentrations ($>3 \times 10^{-8}$ mol/l) leads to an enhanced generation and release of kinins (*e.g.*, Des-Arg^9-bradykinin or other kinin fragments) that are not detected by the radioimmunoassay used (see "Materials and Methods") but are probably due in part to the production of cyclic GMP. This assumption is supported by the finding that in cultured BAEC, an increase in cyclic GMP could be induced to the same extent by either bradykinin or Des-Arg^9-bradykinin and, furthermore, that the effect of both compounds was prevented through icatibant (Wiemer and Wirth, 1992). Thus furosemide seems to cause an enhanced synthesis and release of endothelium-derived kinins, which in turn triggers the formation of the vasodilators nitric oxide and PGI_2 *via* enhancement of cytosolic calcium (Lückhoff *et al.*, 1988).

About the mechanism of action leading to an enhanced synthesis and release of kinins by furosemide, one can speculate that the compound decreases intracellular pH in endothelial cells. In peripheral (Wilkens *et al.*, 1970) and cardiac blood vessels (Noda *et al.*, 1993) under acid conditions, an enhanced release of kinins was observed to occur presumably by the activity of an acid optimum protease (Moshi *et al.*, 1992; Zeitlin *et al.*, 1989), which cleaves kinins from kininogens. The presence of kininogens in endothelial cells was shown by van Iwaarden *et al.* (1988).

Maximal endothelial synthesis of kinins, as well as of cyclic GMP and PGI_2, was reached within a 5-min incubation of the cells with furosemide at concentrations of 3×10^{-7} to 10^{-6} mol/l, which were also observed in human plasma within several minutes after oral administration of 40 mg of furosemide (Rakhit *et al.*, 1987). In contrast, such concentrations of furosemide are insufficient to produce a significant diuresis. However, in the kidney, circulating plasma concentrations of furosemide become concentrated in the loop of the Henle (at least to 10^{-5} mol/l) because of an active secretion of furosemide into the tubular lumen and subsequent fluid reabsorption (Gutsche *et al.*, 1982). Thus the initial vasodilatory effect of furosemide, which already occurs in the absence of extracellular fluid volume loss by diuresis, seems to be in part mediated locally by the stimulation of endothelial nitric oxide and PGI_2 production in resistance and/or capacitance vessels.

The elevation of endothelial nitric oxide and PGI_2 (production) by furosemide might have further relevant biological implications. It has been shown that both autacoids can inhibit platelet aggregation and adhesion (Thiemermann, 1991). In agreement with this hypothesis, an inhibition of ADP-induced platelet aggregation by furosemide has been documented in the human (Kribben *et al.*, 1988). Besides these effects, the elevation of endothelial nitric oxide and PGI_2 production by furosemide might help to prevent the initiation of atherosclerosis. Nitric oxide prevents the adhesion of monocytes to the endothelium, whereas PGI_2 inhibits their chemotactic response (Bath *et al.*, 1991). Additionally, antiatherosclerotic effects of nitric oxide and PGI_2 may result from their respective cyclic nucleotide second-messenger systems, which may inhibit smooth muscle mitogenesis and proliferation (Garg and Hassid, 1989; Thiemermann, 1991).

In conclusion, our findings suggest that the acute hemodynamic effects of furosemide are mediated directly by an enhanced synthesis and release of endothelium-derived kinins, which act as a potent stimulus for the endothelial formation of nitric oxide and PGI_2. Whether the direct effect of furosemide on endothelial cells is significant for the treatment of cardiovascular diseases remains to be determined.

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