In vitro vascular effects of cicletanine in pregnancy-induced hypertension

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1 The vascular effects of cicletanine have been studied *in vitro* on ring preparations of inferior epigastric arteries from normotensive human females and human females with pregnancy-induced hypertension (preeclampsia).

2 Cicletanine (10^{-7} – 10^{-3} M) elicited concentration-dependent relaxation of vessels precontracted with 10^{-7} M noradrenaline (NA) or 60 mM K⁺ but was more potent in the former. Relaxation was significantly greater in rings from preeclamptic patients and was uninfluenced by endothelium removal.

3 The intracellular Ca-dependent contractile responses to 10^{-5} M NA in Ca-free medium as well as the subsequent extracellular Ca-dependent contractions (on restoration of external Ca) were significantly attenuated dose-dependently by cicletanine (10^{-5} M, 3×10^{-4} M) in arterial rings from both normotensive and preeclamptic patients. Cicletanine also relaxed rings precontracted by 25 mM K⁺ but was ineffective against 80 mM K⁺-induced contractions.

4 The inhibition of intracellular Ca-dependent contractions was significantly greater in rings from preeclamptic than from normotensive patients whereas extracellular Ca-dependent contractions were comparably inhibited in both groups. Nifedipine, on the other hand, had little effect on the intracellular Ca-dependent contractions but significantly depressed extracellular Ca-dependent contractions.

5 Cicletanine-induced relaxation was uninfluenced by pretreatment with propranolol, ouabain, tetraethylammonium, procaine, indomethacin, cimetidine or tetrodotoxin but was antagonized by glibenclamide.

6 The results show that cicletanine inhibits contractile responses of human isolated inferior epigastric arteries by a mechanism unrelated to endothelial factors but associated with inhibition of calcium metabolism. An action of cicletanine on glibenclamide-sensitive K⁺ channels is also suggested. Cicletanine-induced inhibition was significantly greater in arteries from preeclamptic patients.

Keywords: Cicletanine; pregnancy-induced hypertension; calcium; human epigastric arteries; vascular smooth muscle; endothelium

Introduction

The vascular effects of cicletanine, an antihypertensive fluoropyridine compound, have been described in a variety of animal (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989; Ebeigbe *et al.*, 1989) and human (Schoeffter & Godfraind, 1988; 1989) smooth muscles. Cicletanine competitively antagonizes histamine H₁-receptor-mediated contractions in the guinea-pig isolated ileum (Schoeffter *et al.*, 1987), rabbit mesenteric artery (Ebeigbe *et al.*, 1989), and human coronary, internal mammary artery and saphenous vein (Schoeffter & Godfraind, 1988; 1989). Also, cicletanine-induced inhibition of contractile responses in rabbit mesenteric arterial and aortic smooth muscles (Ebeigbe *et al.*, 1989) have been associated with interference with calcium metabolism within the vascular smooth muscle cells.

In vitro studies on the effects of cicletanine on arterial smooth muscle contractions in the hypertensive state have concentrated on animal models (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989). The present paper describes our observations on the vascular effects of cicletanine on isolated epigastric arteries from patients suffering from pregnancy-induced hypertension (preeclampsia). Recent reports from our laboratory have shown that the responsiveness of arterial smooth muscles from humans with pregnancy-induced hypertension bears a striking resemblance to observations in vascular smooth muscle from animals with various forms of experimental hypertension: blood vessels from preeclamptic patients appear to possess vascular mem-

brane defects e.g. enhanced activity of the potential-sensitive Ca²⁺ channels (Ebeigbe *et al.*, 1987), attenuated Na-K ATPase enzyme activity (Ebeigbe *et al.*, 1985; Ezimokhai & Ebeigbe, 1985; Ebeigbe & Ezimokhai, 1988) as well as other functional and morphological abnormalities (Aalkjaer *et al.*, 1985). These render the smooth muscle cells hypersensitive to pressor agents as reported for various forms of experimental hypertension (Webb & Bohr, 1981; Aoki *et al.*, 1982).

Methods

Subjects

Two groups of subjects (patients attending the University of Benin Teaching Hospital, Benin City, Nigeria) were studied: (a) Control normotensive women, who in the third trimester of normal pregnancy, delivered following elective or emergency caesarean section as part of their obstetric management; (b) Preeclamptic mothers who were proteinuric and demonstrated persistent elevation of blood pressure to at least 140/90 mmHg in the third trimester of pregnancy, having been normotensive in the first and second trimesters; delivery was also either by elective or emergency caesarean section.

All patients were given general anaesthesia induced by thiopentone with suxamethonium and maintained by a mixture of N₂O and O₂. Transverse suprapubic incision was employed in all patients. Informed consent from the patient as well as approval of the ethical committee of the University of Benin Medical School was obtained to excise a segment of the inferior epigastric artery for the study. On excision, the arteries

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were immediately placed in physiological salt solution (PSS) and transported to the laboratory.

Preparation of tissue

With the aid of dissecting microscope, the arteries were carefully cleaned of adhering connective tissues and cut into rings (internal diameter, 1.5–2.5 mm; 2–3 mm long). The rings were carefully mounted between two L-shaped stainless steel holders in 20 ml organ baths containing PSS. The composition of the PSS was (mM): NaCl 119.0, KCl 4.7, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 24.9, CaCl_2 1.6, CaNa_2EDTA 0.03 and glucose 11.5. The medium was bubbled continuously with a 95% O_2 , 5% CO_2 gas mixture. pH of the solution was 7.4 and temperature was maintained thermostatically at 37°C. Isometric contractions were recorded with Grass FT-03 transducers and the responses were displayed on a Grass model 7 10-channel polygraph. An equilibration period of 2 h was allowed before the start of experiments. Further details of the experimental set-up have been described previously (Ezimokhai & Ebeigbe, 1985; Ebeigbe *et al.*, 1987).

Procedure

Relaxant effect of cicletanine Arterial rings were contracted with 10^{-7} M noradrenaline (NA) or 60 mM K^+ PSS. When the contractile responses were stable, cicletanine was added to the bath cumulatively; the resultant relaxation responses were expressed as a percentage of the initial tension. In other experiments, the relaxant effect of cicletanine was also studied following precontraction induced by 25 or 80 mM K^+ PSS.

Influence of endothelium The relaxant effect of cicletanine following pre-contraction induced by 10^{-7} M NA was examined on intact as well as arterial rings without endothelium. Endothelium removal was effected mechanically (Furchgott & Zawadzki, 1980) by gently rubbing the intimal surface of the vessels. The effectiveness of the procedure for removal of the endothelium was confirmed by the failure of acetylcholine to relax NA (10^{-7} M)-precontracted rubbed arteries.

Intra- and extracellular calcium-dependent contractions Maximal contractile responses to NA (10^{-5} M) were assessed in control PSS and following 15 min exposure to a nominally Ca-free medium. The phasic NA contraction in Ca-free medium was presumed to be due to mobilization of calcium from intracellular stores (Bohr, 1973; Dube *et al.*, 1987; Auguet *et al.*, 1988); whereas restoration of Ca following the intracellular Ca-dependent contraction caused a sustained extracellular Ca-dependent (tonic) component. In Ca-restoration experiments, increases in Ca concentration caused graded contractions, with maximum response at 3.6 mM (not shown); in the results presented a submaximal level (3.0 mM) of Ca was used. The influence of varying concentrations of cicletanine and nifedipine on both components of NA-induced contractions was assessed following application of the antagonists simultaneously with the change to Ca-free PSS (the antagonists were maintained throughout the duration of both components of the contractions).

Influence of drugs on cicletanine-induced relaxation In control experiments, arterial rings from normotensive patients were exposed to 5×10^{-6} M (EC_{50}) cicletanine following NA (10^{-7} M)-induced precontraction. The magnitude of the cicletanine relaxation was estimated as a percentage of the initial NA contraction. The influence of various drugs on the cicletanine relaxation was assessed by applying the agents 15 min before NA stimulation.

The drugs used were: cicletanine hydrochloride (racemate, IHB Research Laboratories, Le Plessis Robinson, France); histamine dihydrochloride (Merck AG, Germany); noradrena-

line bitartrate (Fluka AG, Bucks, Switzerland); propranolol hydrochloride, ouabain, tetraethylammonium chloride, procaine hydrochloride, indomethacin, cimetidine, tetrodotoxin (Sigma); nifedipine (Bayer Leverkusen, FRG). Glibenclamide was a gift from Professor A.H. Weston (Manchester, UK).

Cicletanine was prepared as a stock solution (10^{-1} M) in absolute ethanol and kept at 4°C. Stock solutions of nifedipine (10^{-2} M) were prepared in absolute ethanol and further dilutions made with distilled water. Experiments with nifedipine were carried out in the dark to avoid photoinactivation of the drug. Indomethacin was dissolved in 50 mM Na_2CO_3 . High- K^+ PSS was prepared by equimolar substitution of NaCl with KCl. Ca-free PSS contained no added calcium. All chemicals were of 'analytical reagent' grade.

Statistics

Values are expressed as means $\pm$ s.e.mean, IC_{50} (concentration producing 50% inhibition) values were computed by use of a programme for logit transformation of dose-response curves. Statistical comparison of means was made by Student's *t* test; a *P* value less than 0.05 was considered significant. *n* denotes the number of patients studied; 5 rings were prepared from one arterial segment obtained from each patient.

Results

Relaxant effects of cicletanine

The contractile responses to 10^{-7} M NA and 60 mM K^+ in control (normotensive) arterial rings were 2.8–0.3 g, *n* = 12 and 2.8 ± 0.1 g, *n* = 10, respectively. In rings from preeclamptic patients, contractile responses to both NA (*n* = 12) and 60 mM K^+ (*n* = 9) were enhanced (Table 1). Cicletanine (10^{-7} – 10^{-3} M) relaxed both NA- and 60 mM K^+ precontracted rings concentration-dependently (Figure 1). Arterial rings from preeclamptic patients were significantly more sensitive to cicletanine than those from normotensive (Table 1). In other experiments on arterial rings from normotensive patients (*n* = 3), cicletanine caused concentration-dependent relaxation of the 25 mM K^+ -induced contraction but was ineffective against the 80 mM K^+ -induced contraction (Figure 2).

Figure 1 Concentration-dependent cicletanine-induced relaxation of 10^{-7} M noradrenaline- (open symbols) and 60 mM K^+ - (closed symbols) induced contractions in rings of inferior epigastric arteries from normotensive (circles, *n* = 12) and pregnancy-induced hypertensive (triangles, *n* = 12) patients. Points are means with s.e.mean shown by vertical lines. Asterisks denote significant difference from controls.

Table 1 Magnitudes of contraction (g) in response to 10^{-7} M noradrenaline (NA) and 60 mM K^+ and IC_{50} (M) values for cicletanine-induced relaxation in inferior epigastric arterial rings from normotensive and preeclamptic patients

	10^{-7} M NA		60 mM K^+	
	Normotensive	Preeclamptic	Normotensive	Preeclamptic
Tension	2.8 ± 0.3	$3.2 \pm 0.1^*$	2.8 ± 0.6	$3.2 \pm 0.1^*$
IC_{50}	$3.2(\pm 0.5) \times 10^{-6}$	$1.9(\pm 0.6) \times 10^{-6}^{**}$	$1.8(\pm 1.4) \times 10^{-4}$	$4.1(\pm 0.2) \times 10^{-5}^{**}$
n	12	12	10	9

Means $\pm$ s.e.mean. * $P < 0.001$ and ** $P < 0.05$.

Influence of endothelium

In intact preparations from normotensive patients, 10^{-6} M ACh caused relaxation of 10^{-7} M NA-induced precontraction ($n = 12$) by $78.6 \pm 3.2\%$. ACh-induced relaxation was significantly lower in rings from preeclamptic patients ($46.3 \pm 5.2\%$, $n = 10$).

Contractile responses to 10^{-7} M NA were enhanced following endothelium removal: in rings, from normotensive patients, tension in the presence and absence of endothelium was 2.8 ± 0.2 g ($n = 12$) and 3.3 ± 0.0 g ($n = 9$), respectively; in rings from preeclamptic patients, the respective tension values in the presence and absence of endothelium were 3.0 ± 0.0 g ($n = 10$) and 3.5 ± 0.0 g ($n = 10$).

Relaxation induced by cicletanine (10^{-7} – 10^{-3} M) was uninfluenced by endothelium-removal (data not shown).

Intra- and extracellular Ca^{2+} -dependent contractions

The maximal (10^{-5} M) NA-induced contraction in normal PSS for rings from normotensive ($n = 14$) and preeclamptic ($n = 16$) patients were 3.6 ± 0.1 and 3.8 ± 0.0 g respectively. The magnitude of the phasic, intracellular Ca^{2+} -dependent contraction in rings from normotensive ($n = 14$) and preeclamptic ($n = 16$) patients were 1.0 ± 0.4 and 1.5 ± 0.9 g respectively. The respective extracellular Ca-dependent contraction values for rings from normotensive and preeclamptic patients were 3.9 ± 0.5 and 3.9 ± 0.3 g.

Figure 3 illustrates typical observations on the effect of cicletanine and nifedipine on intra- and extracellular Ca-dependent contractions. Cicletanine (10^{-5} and 3×10^{-5} M) inhibited both intra- and extracellular Ca-dependent contractions in rings from both patient groups. The inhibition of intracellular Ca-dependent contractions was significantly greater in rings from preeclamptic patients (Figure 4) whereas

Figure 2 Cicletanine-induced relaxation in ring preparations of epigastric artery from normotensive patients following precontraction induced by 25 mM (●) or 80 mM (□) K^+ . Means with s.e.mean; $n = 3$.

Figure 3 Representative tracings showing contractile responses of rings of epigastric arteries from normotensive patients to 10^{-5} M noradrenaline (NA) following 15 min exposure to Ca-free PSS in the absence or presence of cicletanine or nifedipine. The phasic contractile response to NA in Ca-free solution and the subsequent tonic contraction on restoration of external calcium represent (respectively), intracellular Ca-dependent and extracellular Ca-dependent contractions.

Figure 4 Summary of experiments described in Figure 3. Cicletanine (10^{-5} , 3×10^{-4} M) attenuated both intracellular (a) and extracellular (b) Ca-dependent contractions in arteries from normotensive (open columns, $n = 14$) and preeclamptic (hatched columns, $n = 16$) patients. Cicletanine-induced inhibition was significantly greater on intracellular Ca-dependent contractions (* $P < 0.001$) and was greater in rings from preeclamptic than from normotensive patients (●, $P < 0.05$).

Figure 5 Attenuation by 10^{-6} M glibenclamide (■, $n = 5$) of cicletanine-induced relaxation following 10^{-7} M noradrenaline precontraction and the lack of effect of 10^{-2} M tetraethylammonium (○, $n = 3$) and 10^{-3} M procaine (△, $n = 3$) in rings from normotensive patients. (●) denotes control ($n = 12$). Means with s.e.mean shown by vertical lines.

the magnitudes of inhibition of extracellular Ca^{2+} -dependent contractions were comparable in rings from normotensive and preeclamptic patients. Contrary to the observation with cicletanine, in rings from normotensive patients, nifedipine (10^{-6} M, $n = 5$) was relatively ineffective against the intracellular Ca^{2+} -dependent contractions whereas the extracellular Ca^{2+} -dependent contractions were inhibited by $79.4 \pm 5.2\%$. The concentration of nifedipine used completely antagonized 60 mM K^{+} -induced contractions (data not shown).

Action of drugs on cicletanine-induced relaxation

The effect of drugs on cicletanine-induced relaxation was studied on arterial rings from normotensive patients. In control experiments ($n = 6$), 5×10^{-6} M cicletanine elicited relaxation of 10^{-7} M NA-induced contractions by $50.4 \pm 3.2\%$.

Exposure of the rings to 10^{-6} M propranolol ($n = 4$), 10^{-5} M ouabain ($n = 3$), 10^{-2} M tetraethylammonium chloride (TEA, $n = 3$), 10^{-3} M procaine ($n = 2$), 10^{-6} M indomethacin ($n = 4$), 10^{-6} M cimetidine ($n = 3$) or 10^{-6} M tetrodotoxin ($n = 3$) did not significantly alter the magnitude of cicletanine-induced relaxation whereas 10^{-6} M glibenclamide ($n = 5$) significantly inhibited the relaxation response; magnitude of relaxation in presence of glibenclamide was $22.3 \pm 2.9\%$. Figure 5 illustrates observations made with K^{+} channel blockers, showing the lack of effect of TEA and procaine and the inhibitory effect of glibenclamide on the concentration-dependent relaxation to cicletanine.

Discussion

The goal of the present study was to characterize the mechanism of cicletanine-induced relaxation of arterial smooth muscle in pregnancy-induced hypertension (preeclampsia). Previous *in vitro* vascular studies on cicletanine in the hypertensive state have employed animal (mainly rat) models (Malherbe *et al.*, 1988; Auguet *et al.*, 1988; Bukoski *et al.*, 1989). Recent work from our laboratory (Ebeigbe *et al.*, 1985; 1986; Ezimokhai & Ebeigbe, 1985; Ebeigbe *et al.*, 1987; Ebeigbe & Ezimokhai, 1988), has shown that studies on vascular smooth muscle in preeclampsia afford an alternative method for assessing vascular disorders in hypertension.

In the present study, we observed that cicletanine-induced relaxation was significantly greater in arteries from preeclamptic

patients than from normotensive patients (Figure 1). The effect of cicletanine was also observed to be greater on NA-precontracted than on K^{+} -precontracted rings. These results suggest a greater susceptibility of arteries from preeclamptic patients to cicletanine action via mechanisms related (perhaps) to receptor activation.

The finding of unchanged cicletanine response following endothelium-removal, clearly shows that the mechanism of action of cicletanine is unrelated to the release of endothelium-derived relaxant factor(s). Comparable lack of endothelial factors in cicletanine-induced relaxation has been reported for rabbit mesenteric arteries (Ebeigbe *et al.*, 1989).

Agonist-induced contractions of vascular smooth muscles can be clearly separable into intracellular- and extracellular Ca^{2+} -dependent components (Bohr, 1973; Bolton, 1979; Ebeigbe, 1982; Dube *et al.*, 1987). The protocol illustrated in Figure 3 permits an assessment of intracellular Ca^{2+} -dependent and extracellular Ca^{2+} -dependent contractions (Dube *et al.*, 1987; Auguet *et al.*, 1988). Whereas the Ca^{2+} entry blocker, nifedipine, had little effect on intracellular Ca^{2+} -dependent and significantly attenuated extracellular Ca^{2+} -dependent contractions (Figure 3), cicletanine attenuated both components of the contractions, concentration-dependently (Figures 3 and 4). The greater inhibitory effect of cicletanine on intracellular Ca^{2+} -dependent contractions in rings from preeclamptic patients and the comparable magnitudes of inhibition of extracellular Ca^{2+} -dependent contractions in rings from both normotensive and preeclamptic patients suggest that the cicletanine action is preferably intracellular. Studies on spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats, in which Fura-2 was used to assess intracellular Ca^{2+} mobilization (Bukoski *et al.*, 1989) have also shown a greater inhibition by cicletanine of intracellular Ca^{2+} release in the SHR.

The differential effect of cicletanine on NA and high- K^{+} contractions is perhaps explicable in terms of their requirements of intra- or extracellular Ca^{2+} pools for tension development: NA mobilizes both Ca^{2+} pools, whereas high- K^{+} contractions are dependent on the extracellular pool (Bolton, 1979; Ebeigbe, 1982). Thus the greater sensitivity of NA-induced contractions to cicletanine inhibition (Figure 1) is probably due to the intracellular action of the drug.

The observations that propranolol, cimetidine, ouabain, indomethacin and tetrodotoxin had no significant effect on cicletanine-induced relaxation suggest that the mode of action of cicletanine is unrelated (respectively) to stimulation of β -adrenoceptors, histamine H_2 -receptors, Na^{+} - K^{+} -ATPase activity, prostaglandin synthesis or stimulation of inhibitory nerves.

Two observations point to a possible involvement of K^{+} channel activation in cicletanine-induced relaxation: the relative ineffectiveness of cicletanine in relaxing 80 mM K^{+} -induced contractions (Figure 2), a criterion for characterizing K^{+} channel activation (Weston, 1990), as well as antagonism by glibenclamide of cicletanine-induced relaxation (Figure 5). However, the two other K^{+} channel blockers tested (TEA and procaine) did not modify cicletanine-induced relaxation, suggesting that a distinct type of glibenclamide-sensitive K^{+} channel mediates the relaxant effect of cicletanine in human epigastric arteries.

In conclusion, the present study shows that cicletanine elicits relaxation of human inferior epigastric arteries. The mechanism of action of cicletanine is unrelated to endothelial factors but is associated with interference with the Ca^{2+} supply, with greater effect on intracellular Ca^{2+} -dependent contractions as well as activation of glibenclamide-sensitive K^{+} channels. Interestingly, the action of cicletanine was more pronounced on arteries from patients with pregnancy-induced hypertension.

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