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# Intimal hyperplasia in human uterine arteries accompanied by impaired synergism between prostaglandin $I_2$ and nitric oxide

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**1** The present experiments were designed to investigate the mechanisms causing intimal hyperplasia in connection with the impaired synergism between prostaglandin $I_2$ ($PGI_2$) and nitric oxide (NO) in human uterine arteries (UAs).

**2** In order to assess the magnitude of intimal hyperplasia, the intima:media ratio (%) was estimated with the aid of an image analyser. Human UAs were classified into two groups, I and II on the basis of the ratio and the degree of elastin deposition of histologically normal specimens. The intima:media ratio in group II was determined to be $38.9 \pm 7.7\%$ ($n=6$), which was significantly ($P<0.01$) higher than that in group I ($16.5 \pm 1.5\%$, $n=7$). Less deposition of elastin was found in group I than in group II.

**3** The relaxation activities of iloprost (IP) as a stable analogue of $PGI_2$ and sodium nitroprusside (SNP) as a NO donor were not different between the two groups. When the minimum concentrations ($C_{min}$) of IP and SNP in producing relaxation were applied together to the UA strips, these compounds interacted synergistically in group I. The observed relaxation ($48.7 \pm 8.8\%$, $n=7$) in this group was significantly ($P<0.01$) greater than the predicted value of $18.8 \pm 3.1\%$ ($n=7$) (the mathematical sum of the relaxations caused by IP and SNP alone). By contrast, these agents interacted in an additive manner in group II. The observed relaxation ($20.8 \pm 9.5\%$, $n=6$) was not significantly different from the predicted value ($18.6 \pm 2.4\%$, $n=6$) in this group.

**4** During the relaxation produced by the addition of IP and SNP alone or in combination, the changes in cyclic nucleotides (cyclic AMP and cyclic GMP) contents (pmol $mg^{-1}$ protein) were assayed. When IP and SNP at $C_{min}$ were applied together to the UA strips, these compounds interacted synergistically in increasing cyclic nucleotides in group I. The observed net increase in the content was determined to be $1.46 \pm 0.30$ ($P<0.05$ *vs.* the predicted value of $0.67 \pm 0.12$) in this group ($n=7$). By contrast, the observed net increase ($0.40 \pm 0.07$, $n=6$) did not exceed the predicted value ($0.65 \pm 0.07$, $n=6$) in group II.

**5** These results suggest that the formation of intimal hyperplasia in group II may be closely related to the impaired synergism between $PGI_2$ and NO in the human UAs.

**Keywords:** Intimal hyperplasia; human uterine artery; cyclic AMP; cyclic GMP; synergism between $PGI_2$ and NO

## Introduction

The mechanism underlying the initiation and/or development of hyperplastic vascular diseases is complex and poorly understood. It is, however, well established that endothelial injury or dysfunction, together with released platelet products, is one of the important mechanisms (Schwartz *et al.*, 1981; Ross, 1986). We have demonstrated that intimal hyperplasia after the endothelial denudation of rabbit carotid arteries results at least partly from the decreased release/production of endothelium-derived relaxing factor [EDRF/nitric oxide (NO)] by the regenerated endothelial cells (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994), and that EDRF/NO is an antiaggregating substance which may also modulate the release of platelet products such as platelet-derived growth factor (PDGF) (Azuma *et al.*, 1986). Garg & Hassid (1989) have provided evidence indicating that the endogenous EDRF/NO functions as an inhibitory modulator of vascular smooth muscle cell mitogenesis and proliferation. Further, it has been reported that prostaglandin $I_2$ ($PGI_2$) inhibits DNA synthesis of vascular smooth muscle cells (Stout, 1982; Morisaki *et al.*, 1988; Uehara *et al.*, 1988).

NO relaxes vascular smooth muscle by activating soluble guanylate cyclase, thus increasing intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Ignarro, 1989). $PGI_2$, on the other hand, exerts its effect through activation of adenylate cyclase, thereby increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Moncada & Vane, 1979). Compounds that increase cyclic AMP and cyclic GMP synergistically inhibit platelet activation (Levin *et al.*, 1982; Radomski *et al.*, 1987; Bowen & Haslam, 1991; Anfossi *et al.*, 1993), and synergistically dilate vascular smooth muscle cells (Maurice *et al.*, 1991; Jang *et al.*, 1993; de Wit *et al.*, 1994). However, as far as we know, there is no report describing whether or not impaired synergism is involved in the occurrence of intimal hyperplasia in human uterine arteries (UAs).

In a previous paper (Azuma *et al.*, 1995b), we have suggested that lack of the production of EDRF/NO by endothelial cells and/or of impaired interaction between EDRF/NO and $PGI_2$ might play an important role in causing intimal hyperplasia in UAs. The present experiments were, therefore, designed to investigate the mechanism causing intimal hyperplasia in connection with impaired synergism between iloprost as a stable analogue of $PGI_2$ (Levitt *et al.*, 1991) and sodium nitroprusside as a NO donor (Kowulak *et al.*, 1992) in human UAs.

## Methods

### *Human uterine arteries (UAs)*

Human UAs were obtained according to the methods described previously (Azuma *et al.*, 1995b), during gynaecological operation from 13 patients (range of age 40 to 51, mean age $45.5 \pm 1.0$ years old) with informed consent for this study. All patients had regular menstrual cycles and were otherwise healthy, normotensive and without any drug treatment. The

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abdominal hysterectomies were carried out within 45 min after clamping the proximal portion of the ascending uterine arteries under thiopentone-sevoflurane anaesthesia. The ascending branches of the uterine arteries in the parametrium were dissected free from the surrounding connective tissues in oxygenated and ice-cold modified Krebs solution. Special care was taken to avoid stretching or other type of damage during operation and dissection. The arteries were cut into transverse strips with endothelium, 7 mm in width, 10 mm in length, and 15 to 25 mg in wet weight.

### *Measurement of mechanical response*

The mechanical responses of UA were measured according to the methods described previously (Azuma *et al.*, 1992; 1995b; Tsujii *et al.*, 1992; Niimi *et al.*, 1994). In brief, a transverse strip was mounted vertically in an organ chamber containing 5 ml of modified Krebs solution, continuously bubbled with 95% $O_2$ and 5% $CO_2$ at 37°C. Special care was taken to avoid unintentional rubbing of the intraluminal surface. One end of each strip was secured to the bottom of the organ chamber and the other end was connected to a force-displacement transducer (TB-611T, Nihon Kohden Kogyo Co.). Isometric changes in tension were recorded on a pen-writing oscillograph (R-64, Rikadenki Kogyo Co.). The length of the strips was adjusted several times until a stable tension of 1 g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period, the solution was replaced every 20 min with fresh solution. The composition of the modified Krebs solution was as follows (mM): NaCl 115.0, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, $KH_2PO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.0.

In one series of experiments, concentration-relaxation curves for iloprost (IP), a stable analogue of prostaglandin $I_2$($PGI_2$)(Levitt *et al.*, 1991), and sodium nitroprusside (SNP), a NO donor (Kowulak *et al.*, 1992), were constructed in a cumulative manner. Cumulatively increasing concentrations of IP and SNP were from $10^{-11}$ M multiplied by 1 or 3 up to $1 \times 10^{-5}$ M, and from $10^{-9}$ M multiplied by 1 or 3 up to $1 \times 10^{-4}$ M, respectively.

The minimum concentration ($C_{min}$) to produce relaxation was obtained from the curve for each UA strip. In another series of experiments, the relaxations in response to IP and SNP at $C_{min}$ alone or in combination were determined in the presence of indomethacin (IM, $10^{-5}$ M) as a cyclo-oxygenase inhibitor (Furchgott & Zawadzki, 1980), $N^G$-nitro-L-arginine (L-NOARG, $10^{-4}$ M) as an inhibitor of NO synthase (Kobayashi & Hattori, 1990) and 3-isobutyl-1-methylxanthine (IBMX, $10^{-5}$ M) as a nonselective inhibitor of cyclic nucleotide phosphodiesterase, in order to eliminate the possible involvement of the endogenous NO and $PGI_2$, since human UAs are capable of producing or releasing NO and $PGI_2$ (Azuma *et al.*, 1995b). IP and/or SNP were added after a stable contraction had been produced by $10^{-6}$ M noradrenaline (NA). Relaxation induced by each vasodilator was expressed as a percentage of the $10^{-6}$ M NA-induced contraction.

### *Measurement of cyclic AMP and cyclic GMP*

The cyclic AMP and cyclic GMP levels were measured according to the method described by Honma *et al.* (1977) and Rapoport & Murad (1983) with minor modifications. IP and SNP at minimum concentrations ($C_{min}$) were added alone or in combination during contraction evoked with $10^{-6}$ M NA. After a 30 min-observation of the relaxation response, the UA strips were quickly removed from the hooks and frozen in 100 μl of 10% trichloroacetic acid (TCA) solution with liquid nitrogen. The UA strips were stored at −80°C until the measurement of cyclic AMP and cyclic GMP. The specimens were minced with scissors and homogenized in 1 ml of 10% TCA with a micro homogenizer (Nissei Biomixer, BM-1, Nihonseiki Kaisha Ltd.). Following centrifugation at 10,000 *g* for 20 min, the supernatant was extracted four times with four volumes of water-saturated ether to remove TCA. After being lyophilized in a centrifugal vaporizer (CVE-100D, Eyela), cyclic AMP and cyclic GMP contents of the dry extract were determined by using radioimmunoassay (RIA) kits (cyclic AMP assay kit and cyclic GMP assay kit) purchased from Yamasa Shoyu Co. (Tokyo, Japan). The amount of protein was determined by use of the protein assay reagent (Bio-Rad).

### *Light microscopy*

Light microscopic examinations were performed according to the method described previously (Azuma *et al.*, 1990; 1992; 1995a,b). The UA specimens were fixed in a 10% neutral formaldehyde solution, then dehydrated with ethanol and embedded in paraffin.

In order to assess intimal hyperplasia, the intima:media ratio (%) was measured with the aid of an image analyser. In brief, a cross section of the UA specimen was stained by the Elastica-Van Gieson (EVG) method and photographed at ×40 magnification. The images were taken into the personal computer by using Photoshop programme. The luminal surface, internal elastica lamina, and the border between medial layer and adventitia were traced and the ratio of the two areas of intima and media were calculated by their dot counts by using an NIH image programme.

Five μm sections were prepared on silanized slides (Dako, Kyoto, Japan). After deparaffinization in xylene and rehydration in graded ethanol, sections were stained with Resorcin-Fuchsin for the detection of elastin.

### *Determination of plasma 17β-oestradiol ($E_2$), total cholesterol and triglyceride*

In order to determine plasma $E_2$ levels, total cholesterol and triglyceride, blood sampling was performed in the early morning on the day before the operation. The plasma concentration of $E_2$ was measured by the specific RIA technique (Lu *et al.*, 1981). The plasma levels of total cholesterol and triglyceride were determined by the cholesterol oxidase (Allain, 1974) and glycerol kinase (Fletcher, 1968) methods, respectively. The normal values in our hospital are 128–249 mg $dl^{-1}$ for total cholesterol and 55–159 mg $dl^{-1}$ for triglyceride.

### *Chemicals*

The following chemicals were used in the present experiments: (−)-noradrenaline bitartrate (NA), sodium nitroprusside (SNP), indomethacin (IM), 3-isobutyl-1-methylxanthine (IBMX) (all from Sigma), $N^G$-nitro-L-arginine (L-NOARG) (Protein Research Foundation). Iloprost as a stable analogue of prostaglandin $I_2$ ($PGI_2$) was a generous gift from Eizai Pharmaceutical Co. Ltd. All chemicals were dissolved in distilled water and kept frozen at −80°C until use ($10^{-2}$ M stock solution) except for L-NOARG, iloprost and indomethacin, which were dissolved in dimethylsulphoxide (DMSO, Art. 2950, Merck). DMSO was present in a final concentration of 0.1% in the experiments with these agents, and this concentration had no effect on any variables tested.

### *Calculation and statistical analysis*

All data were expressed as means ± s.e.mean. The statistical significance of difference between the two means was determined by Student's *t* test. Differences were considered significant if $P<0.05$.

## Results

### *Baseline data on the patients*

As described below, the human uterine arteries (UAs) could be classified into two groups by the different magnitude of the

intima:media ratio. The baseline data of the patients in groups I and II are shown separately in Table 1. The mean age, plasma 17β-oestradiol ($E_2$), total cholesterol and triglyceride levels were not significantly different from each other between the two groups.

### *Morphological findings*

In order to assess the magnitude of intimal hyperplasia, the intima:media ratio (%) was determined with the aid of the image analyser. In a previous paper (Azuma *et al.*, 1995b), we reported that 14 UA specimens out of 24 isolated from 24 patients were histologically normal. The intima:media ratio (%) of these 14 preparations was estimated to be 19.6±4.5 (mean±standard deviation). The 95% confidence limits of the mean value were calculated as 17.2–22.0. Thus, we considered a ratio less than the upper value of the 95% confidence limits as normal. In addition to a lower media:intima ratio, less deposition of elastin was noticed in 14 histologically normal specimens (Azuma: unpublished observations). On the basis of these criteria, human UAs could be classified into two groups. The ratio in group II was estimated to be 38.9±7.7% (range: 24.2–72.3, $n=6$), which was significantly ($P<0.01$) greater than that in group I (16.5±1.5%, range: 11.6–22.0, $n=7$) (Figure 1). In contrast, the medial layer remained unaltered both in groups I and II. The representative histological findings of human UA specimens are shown in Figure 2.

Extracellular matrix formation is one of the important factors contributing to the development of intimal hyperplasia (Strauss *et al.*, 1994). We therefore compared elastin deposition in the intimal area between groups I and II. The elastin

**Table 1** Baseline data of the patients

| | *Group I* | *Group II* |
|---|---|---|
| Age (years old) | 45.0±1.5 (7) [40–51] | 46.0±1.3 (6) [41–49] |
| 17β-Oestradiol (pg ml$^{-1}$) | 55.0±19.8 (7) [5.0–159.3] | 17.0±8.2 (6) [0.0–52.0] |
| Total cholesterol (mg dl$^{-1}$) | 172.3±8.9 (7) [153–221] | 175.7±10.5 (6) [146–214] |
| Triglyceride (mg dl$^{-1}$) | 82.7±14.5 (7) [57.0–92.0] | 78.5±3.7 (6) [67.0–92.0] |

Results are given as mean±s.e.mean. Figures in parentheses and square brackets indicate the number of patients and the range of data, respectively. The normal values in our hospital are 128–249 mg dl$^{-1}$ for total cholesterol and 55–159 mg dl$^{-1}$ for triglyceride.

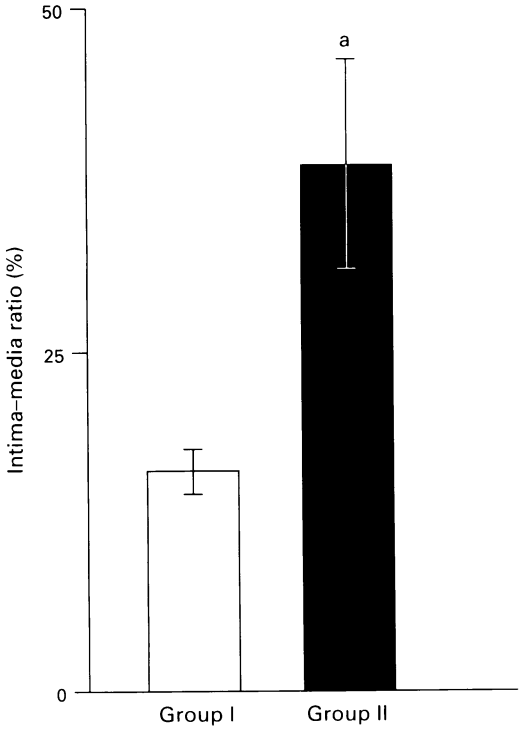


**Figure 1** Comparison of the intima – media ratio (%) in groups I and II. Human UAs were classified into two groups by the different magnitude of the intima – media ratio (%). The ratio in group II was estimated to be 38.9±7.7% ($n=6$), which was significantly ($P<0.01$) greater than that in group I (16.5±1.5%, $n=7$). [a]: Significant difference at $P<0.01$ *vs.* group I.

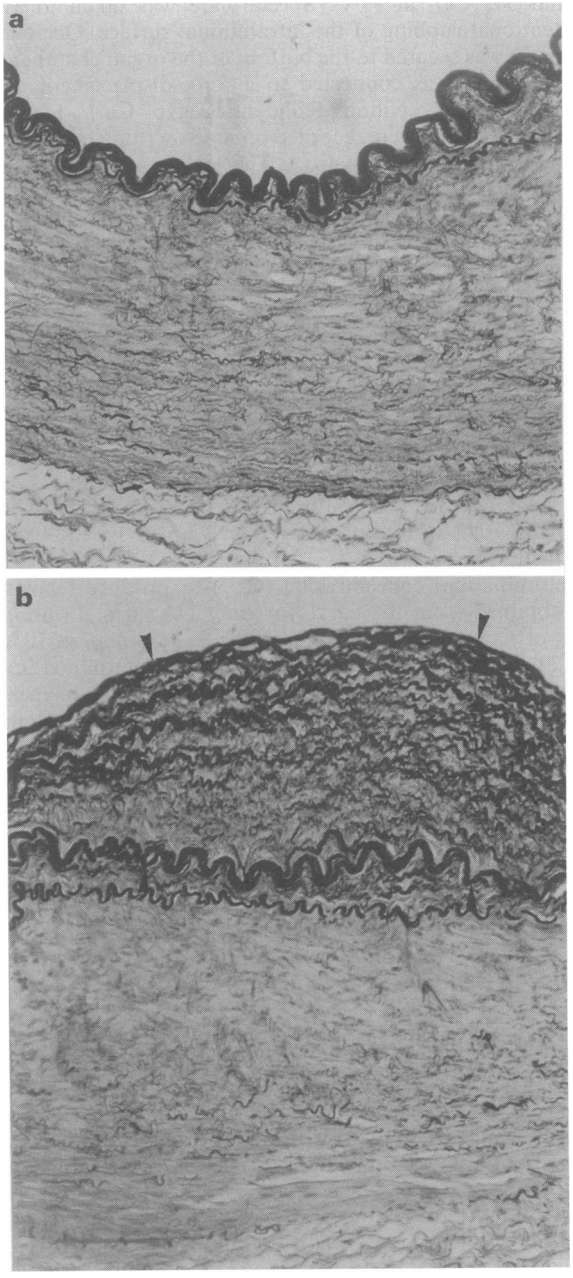


**Figure 2** Representative histological findings in human uterine artery specimens. (a) Almost normal appearance (Elastica-Van Gieson staining, original magnification × 200) (I.R., 40 years old in group I). (b) Severe intimal hyperplasia can be observed (Elastica-Van Gieson staining, original magnification × 200) (O.I., 49 years old in group II). Arrow heads indicate thickened intima.

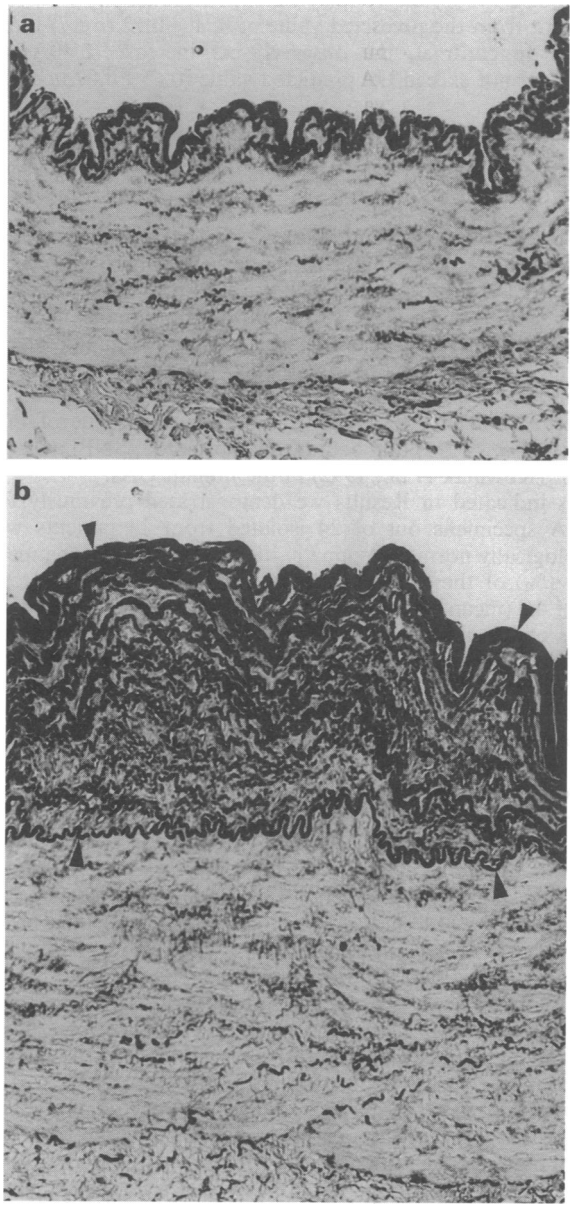


**Figure 3** Comparison of the elastin deposition in intimal area between groups I and II. (a) Almost normal appearance (Resorcin-Fuchsin staining, original magnification × 200) (I.R., 40 years old in group I). (b) Marked elastin deposition and severe intimal thickening can be observed (Resorcin-Fuchsin staining, original magnification × 200) (O.I., 49 years old in group II). Arrow heads indicate thickened intima.

deposition were clearly different between groups I and II. As shown in Figure 3, less elastin deposition was found in group I than in group II.

### *Relaxation response of the UA strips to iloprost (IP) and sodium nitroprusside (SNP)*

IP ($10^{11}-10^{-5}$ M) and SNP ($10^{-9}-10^{-4}$ M) produced a concentration-dependent relaxation of UA strips in groups I and II, which had been contracted with $10^{-6}$ M noradrenaline (NA). The potency of IP and SNP in producing relaxation was not different in the two groups. In order to test the synergism between IP and SNP, the minimum concentrations ($C_{min}$, −log M) that produced relaxation were obtained from the concentration-relaxation curves for these two agents. The $C_{min}$ in groups I and II were determined to be $9.71\pm0.81$ ($n=7$) and $9.75\pm1.17$ ($n=6$) for IP, respectively, and $8.57\pm0.13$ ($n=7$) and $8.67\pm0.17$ ($n=6$) for SNP, respectively (Table 2). These values were not significantly different from each other between the two groups. The magnitude of relaxation produced at $C_{min}$ in groups I and II was $8.2\pm2.5\%$ ($n=7$) and $12.0\pm3.0\%$ ($n=6$) for IP, respectively, and $12.6\pm3.2\%$ ($n=7$) and $8.6\pm1.8\%$ ($n=6$) for SNP, respectively. Again, these values were not significantly different from each other between the two groups. The predicted relaxations (the mathematical sum of the relaxations) caused by IP and SNP alone were calculated to be $18.8\pm3.1\%$ ($n=7$) for group I and $18.6\pm2.4\%$ ($n=6$) for group II.

When the $C_{min}$ of IP and SNP were applied together to the UA strips, these compounds interacted synergistically (supra-additively) in group I. The observed relaxation ($48.7\pm8.8\%$, $n=7$) in group I was significantly ($P<0.01$) greater than the predicted value ($18.8\pm3.1\%$, $n=7$) in this group, whereas, these agents interacted in an additive manner in group II. The observed relaxation ($20.8\pm9.5\%$, $n=6$) was not significantly different from the predicted value ($18.6\pm2.4\%$, $n=6$) in this group (Figure 4).

### *Changes in cyclic nucleotides levels caused by iloprost (IP) and sodium nitroprusside (SNP)*

As shown in Table 2, the basal levels of cyclic AMP and cyclic GMP in the human UA specimens without addition of any agent showed no significant difference between the two groups. During the relaxation produced by IP and SNP alone or in combination, the changes in cyclic nucleotides levels were determined. Thirty min after a single addition of the minimum concentrations ($C_{min}$) of IP or SNP in producing relaxation, the net increases in the cyclic AMP and cyclic GMP contents were slight in the two groups. Again, these values were not significantly different between the two groups (Table 2).

Changes in cyclic AMP and cyclic GMP are shown in Table 3 when $C_{min}$ of IP and SNP in producing relaxation were applied together to the UA strips. The observed net increase in

**Table 2** Comparison of minimum concentration ($C_{min}$) to produce relaxation, basal level and net increase in cyclic nucleotides between groups I and II

| | | | | *Basal level* (pmol mg$^{-1}$ protein) | | *Net increase* (pmol mg$^{-1}$ protein) | |
|---|---|---|---|---|---|---|---|
| *Group* | *Agent* | n | $C_{min}$ (-log M) | *Cyclic AMP* | *Cyclic GMP* | *Cyclic AMP* | *Cyclic GMP* |
| Group I | IP | 7 | $9.71\pm0.81$ | $2.17\pm0.24$ | $0.46\pm0.07$ | $0.36\pm0.04$ | $0.06\pm0.05$ |
| | – | | – | | | – | – |
| | SNP | | $8.57\pm0.13$ | | | $0.03\pm0.03$ | $0.22\pm0.08$ |
| Group II | IP | 6 | $9.75\pm1.17$ | $2.34\pm0.21$ | $0.42\pm0.06$ | $0.40\pm0.08$ | $0.03\pm0.02$ |
| | – | | – | | | – | – |
| | SNP | | $8.67\pm0.17$ | | | $0.05\pm0.03$ | $0.17\pm0.03$ |

Results are given as mean ± s.e.mean. Iloprost (IP) or sodium nitroprusside (SNP) at minimum concentration ($C_{min}$) to produce relaxation was added alone during contraction evoked with $10^{-6}$ M noradrenaline (see text).

group I was significant ($P<0.05$) for cyclic AMP and tended to be enhanced but not significant for cyclic GMP *vs.* the corresponding predicted value (the mathematical sum of the net increase with IP or SNP alone). By contrast, the observed net increases in cyclic AMP and cyclic GMP in group II did not exceed the corresponding predicted values (Table 3). When the changes are expressed as cyclic AMP plus cyclic GMP, the interaction between IP and SNP was more prominent. As shown in Figure 5, these compounds interacted synergistically (supra-additively) in increasing cyclic nucleotides in group I. The observed net increase in the content was determined to be $1.46\pm0.30$ ($n=7$) in group I, which is significantly ($P<0.05$) different from the predicted value of $0.67\pm0.12$ ($n=7$) in this group. In contrast, the observed net increase ($0.40\pm0.07$, $n=6$) did not exceed the predicted value ($0.65\pm0.07$, $n=6$) in group II.

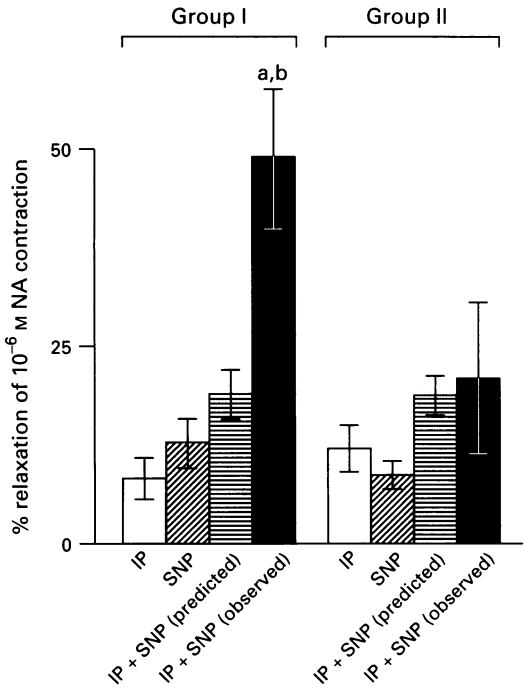


**Figure 4** Comparison of the relaxation responses to iloprost (IP) and sodium nitroprusside (SNP) at minimum concentrations ($C_{min}$) alone or in combination. The $C_{min}$ ($-\log$ M) in groups I and II were determined to be $9.71\pm0.81$ ($n=7$) and $9.75\pm1.17$ ($n=6$) for IP, respectively, and $8.57\pm0.13$ ($n=7$) and $8.67\pm0.17$ ($n=6$) for SNP, respectively. The magnitude of relaxation produced at $C_{min}$ in groups I and II was $8.2\pm2.5\%$ ($n=7$) and $12.0\pm3.0\%$ ($n=6$) for IP, respectively, and $12.6\pm3.2\%$ ($n=7$) and $8.6\pm1.8\%$ ($n=6$) for SNP, respectively. When the $C_{min}$ of IP and SNP were applied together to the UA strips, the observed relaxation ($48.7\pm8.8\%$, $n=7$) in group I was significantly ($P<0.01$) greater than the predicted value (the mathematical sum of the relaxation with IP and SNP alone; $18.8\pm3.1\%$, $n=7$). The observed relaxation ($20.8\pm9.5\%$, $n=6$) in group II was not significantly different from the predicted value ($18.6\pm2.4\%$, $n=6$). [a]$P<0.01$ *vs.* the predicted value in group I. [b]$P<0.05$ *vs.* the observed value in group II.

## Discussion

In a previous paper (Azuma *et al.*, 1995b), we suggested that the lack of the production of endothelium-derived relaxing factor {EDRF/nitric oxide (NO)} and/or of the synergism between $PGI_2$ and EDRF/NO might play an important role in the formation of intimal hyperplasia in the human uterine arteries (UAs). Thus, we investigated the mechanism causing the intimal hyperplasia in connection with the impaired synergism between iloprost (IP) as a stable analogue of $PGI_2$ (Levitt *et al.*, 1991) and sodium nitroprusside (SNP) as a NO donor (Kowulak *et al.*, 1992) in the human UAs.

As indicated in Results we demonstrated previously that 14 UA specimens out of 24 isolated from 24 patients were histologically normal (Azuma *et al.*, 1995b). The intima:media ratio (%) of these 14 preparations has been estimated to be $19.6\pm4.5$ (mean $\pm$ standard deviation) and the 95% confidence limits of the mean value were calculated as 17.2–22.0. Thus, we considered a ratio of less than the upper value of the 95% confidence limits as normal. In addition to lower intima:media ratio, less deposition of elastin was noticed in 14 histologically normal specimens (Azuma: unpublished observations). On the basis of these criteria, the human UAs of the present study were classified into two groups, I and II. The intima:media ratio in group II was estimated to be $38.9\pm7.7\%$ ($n=6$), which was significantly ($P<0.01$) higher than that in group I ($16.5\pm1.5\%$, $n=7$) (Figure 1). Elastin is one of the major fibrous proteins of vascular extracellular matrices and its synthesis has been shown to increase in the thickened intima after arterial injury (Swedberg *et al.*, 1989; Snow *et al.*, 1990; Strauss *et al.*, 1994). Comparing the elastin deposition in the intimal area between groups I and II, less elastin deposition was found in group I than in group II. We therefore suggest that the higher media:intima ratio in group II is closely related to the higher elastin deposition in this group.

The mean age, plasma 17$\beta$-oestradiol ($E_2$), total cholesterol and triglyceride levels were not significantly different from each other between the two groups (Table 1). Thus, the different magnitude of the intimal hyperplasia between the two groups could not be explained by changes in these parameters.

NO relaxes vascular smooth muscle cells by activating soluble guanylate cyclase, thus increasing intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Ignarro, 1989). $PGI_2$, on the other hand, exerts its effect by activation of adenylate cyclase, thereby increasing adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Moncada & Vane, 1979). Compounds that increase cyclic AMP and cyclic GMP synergistically inhibit platelet activation, (Levin *et al.*, 1982;

**Table 3** Combination effects of iloprost (IP) and sodium nitroprusside (SNP) on the increase in cyclic nucleotides

| *Group* | *Combination* | n | | *Net increase in cyclic nucleotides* (pmol mg$^{-1}$ protein) *Cyclic AMP* | *Cyclic GMP* |
|---|---|---|---|---|---|
| Group I | IP + SNP | 7 | Observed | $0.73\pm0.14$[a] | $0.74\pm0.35$ |
| | | | Predicted | $0.39\pm0.05$ | $0.28\pm0.09$ |
| Group II | IP + SNP | 6 | Observed | $0.28\pm0.06$ | $0.12\pm0.03$ |
| | | | Predicted | $0.45\pm0.06$ | $0.20\pm0.03$ |

Results are given as mean $\pm$ s.e.mean. Predicted value was calculated as sum of the net increase with IP or SNP alone. [a]Significant difference *vs.* corresponding predicted value at $P<0.05$. Iloprost (IP) or sodium nitroprusside (SNP) at minimum concentration ($C_{min}$) to produce relaxation was added in combination during contraction evoked with $10^{-6}$ M noradrenaline (see text).

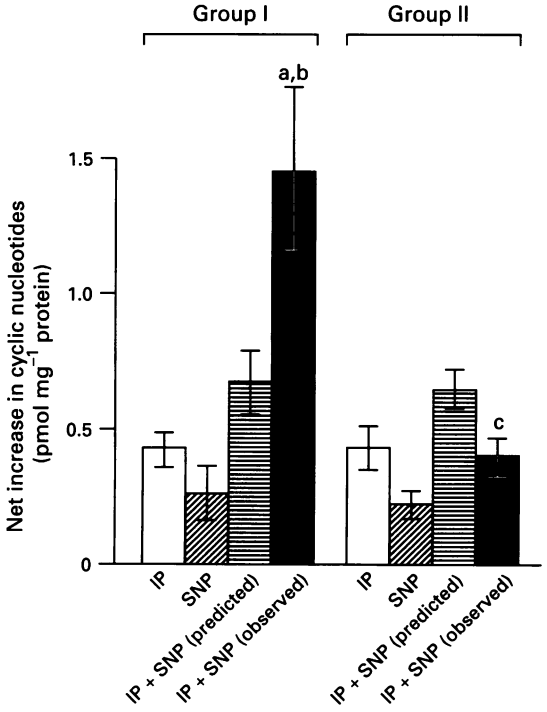


**Figure 5** Changes in cyclic nucleotides levels induced by iloprost (IP) and sodium nitroprusside (SNP) at minimum concentrations ($C_{min}$) alone or in combination. The basal levels of cyclic AMP and cyclic GMP in the human UA specimens without addition of any agent were determined to be $2.17 \pm 0.24$ ($n=7$) and $0.46 \pm 0.07$ ($n=7$) pmol mg$^{-1}$ protein in group I, respectively, and $2.34 \pm 0.21$ ($n=6$) and $0.42 \pm 0.06$ ($n=6$) pmol mg$^{-1}$ protein in group II, respectively. During the relaxation produced by IP and SNP alone or in combination, the changes in cyclic nucleotides levels were determined. Results were given as cyclic AMP plus cyclic GMP (pmol mg$^{-1}$ protein). Thirty min after a single addition of the minimum concentrations ($C_{min}$) of IP or SNP in producing relaxation, the net increase in the cyclic nucleotides contents were $0.42 \pm 0.06$ ($n=7$) with IP and $0.26 \pm 0.10$ ($n=7$) with SNP in group I, and $0.43 \pm 0.08$ ($n=6$) with IP and $0.22 \pm 0.05$ ($n=6$) with SNP in group II. When $C_{min}$ of IP and SNP in producing relaxation were applied together to the UA strips, these compounds interacted synergistically (supra-additively) in increasing cyclic nucleotides in group I. The observed net increase in the content was determined to be $1.46 \pm 0.30$ ($n=7$) in group I, which was significantly ($P<0.05$) different from the predicted value (the mathematical sum of the net increase with IP and SNP alone) of $0.67 \pm 0.12$ ($n=7$) in this group. In contrast, the observed net increase ($0.40 \pm 0.07$, $n=6$) in group II did not exceed the predicted value ($0.65 \pm 0.07$ $n=6$). $^{a}P<0.05$ *vs.* the predicted value in group I; $^{b}P<0.01$ *vs.* the observed value in group II; $^{c}P<0.01$ *vs.* the predicted value in group II.

Radomski *et al.*, 1987; Bowen & Haslam, 1991; Anfossi *et al.*, 1993), and synergistically dilate vascular smooth muscle cells (Maurice *et al.*, 1991; Jang *et al.*, 1993; de Wit *et al.*, 1994). This synergistic interaction seems to be specific for compounds that increase cyclic AMP and cyclic GMP, since no synergism between $PGI_2$ and cromakalim as a cyclic GMP-independent vasodilator (Newgreen *et al.*, 1990) was observed in dilating blood vessels (de Wit *et al.*, 1994). The relaxation activities of iloprost (IP) as a stable analogue of $PGI_2$ and sodium nitroprusside (SNP) as a NO donor were not different in the UA strips of both groups, which had been contracted with noradrenaline. The magnitude of relaxation produced at minimum concentration ($C_{min}$) in groups I and II was not significantly different between the two groups. These findings indicate that the ability of the underlying smooth muscle to relax remained unchanged in the two groups. However, when the $C_{min}$ of IP and SNP were applied together to the UA strips, these compounds interacted synergistically in producing relaxation in group I, but in an additive manner in group II (Figure 4). Further, when the $C_{min}$ of IP and SNP in producing relaxation were applied together to the UA strips, these compounds interacted synergistically in increasing cyclic nucleotides in group I. Thus, it appears likely that, in group I, the synergistic increase in cyclic nucleotides contents with the $C_{min}$ of IP and SNP was responsible for the synergism in producing relaxation. By contrast, the synergistic interaction between IP and SNP seems to be impaired in the UAs of group II.

The precise reason why the synergistic interaction between $PGI_2$ and NO was observed only in group I remains unclear from the present experiments. Jang *et al.* (1993) reported that isoprenaline and atriopeptin II acted synergistically to inhibit the phenylephrine-induced contraction of aortic smooth muscle. They suggested that cyclic GMP, formed by the action of atriopeptin II on receptor guanylate cyclase, may inhibit aortic cyclic nucleotide phosphodiesterase III and that an increased accumulation of cyclic AMP mediates the observed synergism. However, this possibility seems to be ruled out in the present case, since our experiments were performed in the presence of a comparatively high concentration of 3-isobutyl-1-methylxanthine (IBMX) as a non-selective inhibitor of cyclic nucleotide phosphodiesterase. The mechanisms producing synergism in group I is now under investigation in our laboratory.

We have demonstrated that the intimal hyperplasia after the endothelial denudation results at least partly from the decreased production of EDRF/NO by the regenerated endothelial cells (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994), and that EDRF/NO is an antiaggregating substance which may also modulate the release of platelet products such as platelet-derived growth factor (PDGF) (Azuma *et al.*, 1986). According to Garg & Hassid (1989), the endogenous EDRF/NO functions as an inhibitory modulator of vascular smooth muscle cell mitogenesis and proliferation. Further, it has been reported that $PGI_2$ inhibits DNA synthesis of vascular smooth muscle cells (Stout, 1982; Morisaki *et al.*, 1988; Uehara *et al.*, 1988). In addition to the facts described above, NO and/or $PGI_2$ are involved in producing relaxation of the human UA strips (Azuma *et al.*, 1995b). If these findings are considered together, it is reasonable to conclude that the formation of intimal hyperplasia in group II may be closely related to the impaired synergism between NO and $PGI_2$ in these human UAs.

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