Br. J. exp. Path. (1985) 66, 399–408

Effect of hypoxia and carbon monoxide on collagen synthesis in cultured porcine and bovine aortic endothelium

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Received for publication 28 September 1984

Summary. The cell layers and medium of cultured porcine and bovine aortic endothelium have been examined to test the effects of 24 h treatment with two factors associated with cigarette smoke—hypoxia and carbon monoxide, on cell numbers, total protein including collagen/10^6 cells, collagen type profile and ultrastructure. The most significant findings were that the responses varied with the species and that the effects on protein synthesis including collagen differed depending on the nature of the insult; in general, moreover carbon monoxide tended to reverse the action of hypoxia, a finding supported by ultrastructural evidence. The phenotypic collagen profiles were unaffected by either hypoxia or carbon monoxide.

Keywords: endothelium, aorta, hypoxia, carbon monoxide, collagen, ultrastructure

Cultured porcine aortic endothelium synthesizes various interstitial collagens in addition to basement membrane (Levene & Heslop 1977; Barnes *et al.* 1978) suggesting that collagen found in atheromatous plaques may have derived in part from endothelium as well as from smooth muscle cells (Ross & Glomset 1973).

Our aim has been to attempt to define a primary lesion in endothelium which may lead eventually to the formation of an atheromatous plaque. Hypoxia was selected as a potentially atherogenic insult since cigarette smoking is considered to be one of the three major risk factors in the development of myocardial infarction. Hypoxia in smoking is caused by the formation of carboxyhaemoglobin in the blood, due to the presence of carbon monoxide in cigarette smoke, but carbon monoxide itself can also be directly injurious to the vascular system (Astrup *et al.* 1970).

This study is a biochemical and ultrastructural comparison of the effects of hypoxia and carbon monoxide on cultured porcine and bovine aortic endothelium; the results indicate that their responses differ—there is clearly a species difference.

Methods

Tissue culture. Aortic endothelial cells were prepared from pig aorta which had been obtained from an abattoir, by a method devised by Leake & Bowyer (1981). In essence, this consisted of maintaining sterility by clamping both ends and ligating branches, and then incubating the aorta at 37°C in a sterile bath of Hank's solution, the aortic lumen having been filled before clamping with a 0.2% solution of collagenase (Sigma I). The released endothelial cells were plated after washing, in 60 mm Falcon dishes, usually at a concentration of 50 000 cells in 4 ml of medium per dish and were fed twice weekly; the medium used was Dulbecco and Vogt's modification of minimal Eagle's medium, supplemented with 20%

fetal calf serum (Flow Laboratories, Scotland), and gassed with a mixture of 75% N_2, 20% O_2 and 5% CO_2.

Bovine aortic endothelium was obtained from Dr Mary Daniels of the Douglas Cancer Research Centre, Clatterbridge Hospital, Liverpool.

Histology. Cells were grown on cover slips in 35-mm Falcon tissue culture dishes, fixed in methanol after washing in phosphate-buffered saline (PBS) and stained with the May-Grünwald modification of the Giemsa stain.

Electron microscopy. The cultures were fixed, dehydrated and embedded directly in the culture dishes as follows: cultures were rinsed twice with PBS, then were fixed by flooding the dishes with 2.5% glutaraldehyde in 0.1% cacodylate buffer plus 10% $CaCl_2$, pH 7.4, at room temperature for 30 min.

After three washes with buffer, the cultures were post-fixed for 20 min in 1% osmium tetroxide in the same buffer. After three further washes, the cells were dehydrated using a series of graded ethanol solutions.

Gradually cells were embedded in Spurr resin (Spurr 1969) by mixing resin directly with absolute alcohol. After allowing penetration and polymerization of the resin, for 16 h at 60°C, the plastic of the dish was detached from the resin by immersion in liquid nitrogen. The specimens were cut at right angles or parallel to the plane of the dishes. Sections were cut on an LKB ultramicrotome, stained with uranyl acetate followed by lead citrate and viewed with a Phillips electron microscope, operated at 80 kV.

DNA estimations. For each analysis three 60 mm plates were used, and the average DNA estimated (Burton 1956) to give the cell numbers per plate.

Protein estimation. For each gaseous phase three estimations of the total protein were made from one 60 mm dish using the method of Lowry *et al.* (1951).

Collagen content. For each measurement, two plates were prelabelled for 24 h with 20 μCi L-[5-^{3}H]-proline (Amersham International) per plate and ascorbic acid at 50 μg/ml concentration. Each plate yielded 4 ml medium. Cell layers were washed in 1 ml distilled water, scraped from the dishes and pooled. Samples were heated for 10 min at 100°C to destroy any endogenous proteolytic enzymes and were then extensively dialysed against 1 M Tris, 0.01 M $CaCl_2$ pH 7.6 at 4°C. They were digested in a dialysis bag containing 20 units/ml of purified, highly specific Worthington collagenase (CLSPA), demonstrably free of all other proteolytic enzymes, and simultaneously dialysed by immersing the dialysis bag in 10 ml Tris/-$CaCl_2$ buffer in a screw cap tube. Conditions used were 24 h at 25°C in a shaking water bath. Samples of all diffusates were taken for counting, which was performed in vials containing phase combining scintillant (PCS) in a Beckman LS-250 liquid scintillation counter. The non-diffusable fractions were hydrolysed in 6 N HCl for 24 h at 100°C, then evaporated to dryness and redissolved in water. A sample of each was removed for counting.

It was thus possible to measure both total protein synthesis as well as to calculate the percentage and absolute amount of collagen synthesized, during the 24-h labelling period.

Collagen typing. During the 24-h experimental period when cultures were subjected to changes in their gaseous environment, each plate received 100 μCi L-[5-^{3}H]-proline (Amersham International) plus 50 μg ascorbic acid/ml medium, plus 100 μg/ml medium of β-aminoproprionitrile; the latter was added to increase the yield of extractable collagen for typing.

Collagens were isolated and the relative propations of collagen types I, III and V

assayed by SDS-PAGE (Laemmli 1970; Sykes *et al.* 1978) and fluorography (Bonner & Laskey 1974; Laskey & Mills 1975) as previously described (Levene *et al.* 1984).

Production of hypoxic environment. The Fildes' jars containing the experimental dishes were gassed for the appropriate time with a mixture of 95% N_2 and 5% CO_2. This mixture is known to contain traces of O_2 and is thus hypoxic rather than anoxic. The control plates received the normal mixture of 75% N_2, 20% O_2 and 5% CO_2.

Production of carbon monoxide environment. After gassing the Fildes' jar containing the experimental plates with the usual 75% N_2, 20% O_2 and 5% CO_2 mixture or with the 95% N_2, 5% CO_2 mixture, the approximate volumes of carbon monoxide were injected into the Filde's jars by means of syringes which had been filled with pure carbon monoxide and allowed to equilibrate to atmospheric pressure before injection. The syringes were sealed during equilibration to avoid entry of atmospheric oxygen. Following a number of preliminary experiments to decide the final concentration of carbon monoxide to be used, we eventually decided to use 80 ml of carbon monoxide per 3-litre Fildes' jar for 24 h since this appeared to produce the maximal biochemical effect on protein synthesis with the minimal amount of cell death.

Results

Histology

No obvious differences were noted as a result of the treatments to which the cells had been subjected.

Electron microscopic findings in porcine and bovine cell layers following hypoxia and/or carbon monoxide treatment

Controls. In both porcine and bovine cells, the controls show a normal arrangement and structure of cytoplasmic organelles including mitochondria, free ribosomes, Golgi apparatus and rough endoplasmic reticulum (RER); in the extracellular spaces fibrous material is associated with the plasma membrane. (Figs 1*a* and 2*a*).

Hypoxia. Hypoxia induces cell damage; there is a reduction in the number of free ribosomes, degranulation of RER and accumulation of dense lysosomal bodies and of myelin figures. Extracellular filamentous and granular material is still present. (Figs 1*b* and 2*b*).

Carbon monoxide. In normoxic cells treated with carbon monoxide, the cell ultrastructure is well preserved, especially the mitochondria and the synthetic apparatus i.e. ribosomes and RER. Occasionally myelin figures are present. Filamentous material is easily recognizable outside the plasma membrane. (Figs 1*c* and 2*c*).

Hypoxia plus carbon monoxide. Cells treated with hypoxia plus carbon monoxide show some degenerative features—accumulation of dense lysosomal bodies and of myelin figures, and swelling of smooth ER, but mitochondria show normal configuration and RER is largely preserved. Extracellular spaces are filled with relatively abundant filamentous material especially in the bovine endothelial cell layers. (Figs 1*d* and 2*d*).

Effect of hypoxia and/or carbon monoxide on porcine and bovine cell numbers

Hypoxia depressed the cell counts in both species. However, whilst carbon monoxide increased the hypoxic effect in the porcine cell numbers, it appeared to somewhat counteract the effect in the bovine cell numbers. (Table 1).

Effect of hypoxia and/or carbon monoxide on total protein in procine and bovine cell layer per 10^6 cells

Chemical analysis of the cell layer by the

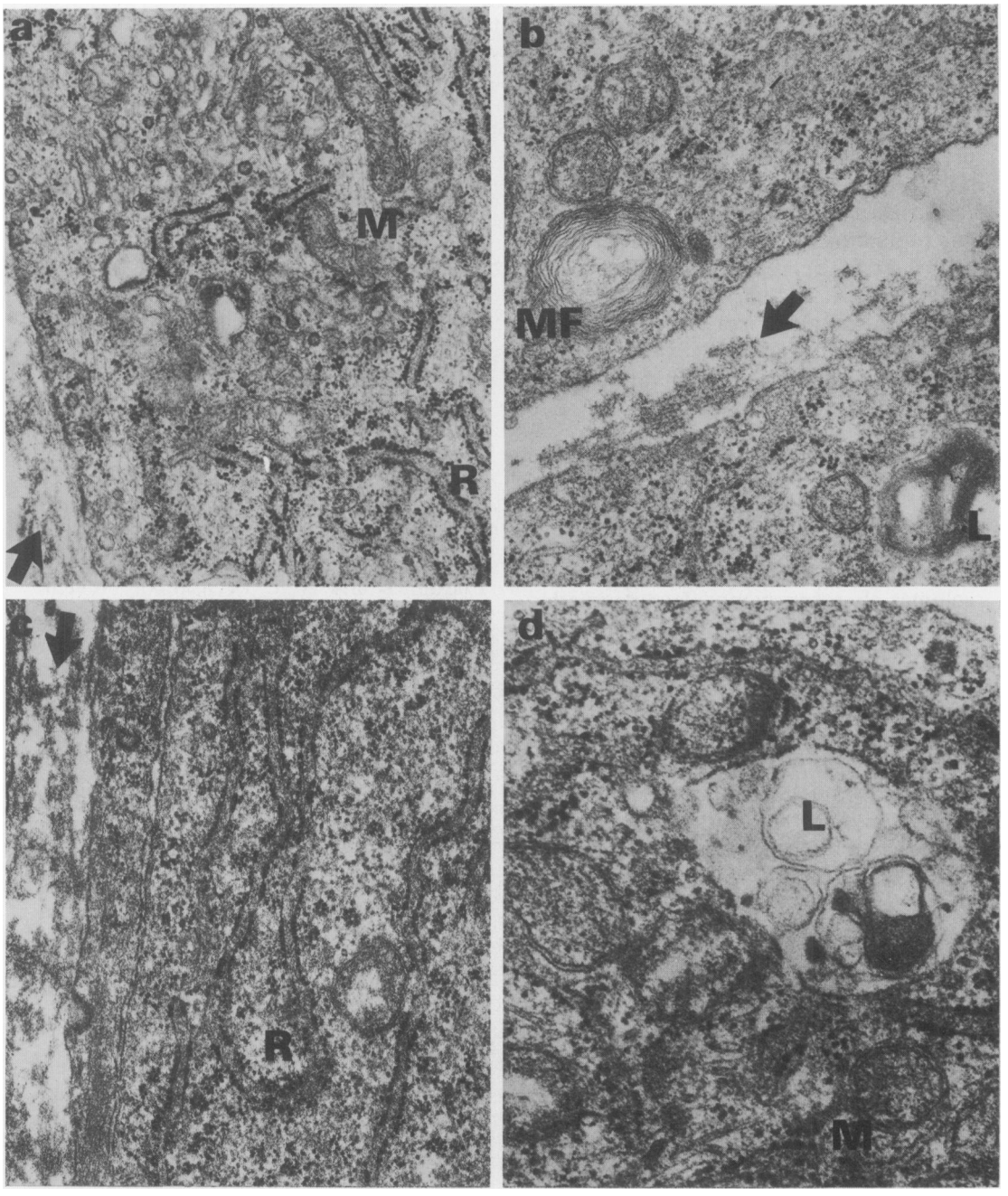

Fig. 1. Pig endothelial cells. (*a*) Control (normoxic) pig endothelial cells. Cells show normal arrangement of the cytoplasm, mitochondria (M) numerous cisternae of RER (R), polysomes and extracellular granular material is present (arrow). × 23 000. (*b*) Hypoxic pig endothelial cells. Cytoplasm shows some degenerative features—myelin figures (MF) and dense lysosomal bodies (L); RER is disorganized and some mitochondria show swelling. Extracellular fibrils are visible (arrow). × 25 000. (*c*) Normoxic pig endothelial cells plus carbon monoxide. Cytoplasm is largely filled by polysomes and RER (R); degenerative features are not present. Granular and filamentous material is present in extracellular spaces between the cells (arrow). × 24000. (*d*) Hypoxic pig endothelial cells plus carbon monoxide. Mitochondria (M) are mainly well preserved and RER and polysomes are largely still present. Lysosomes (L) are filled by electron-dense bodies and membrane residues. × 59 000.

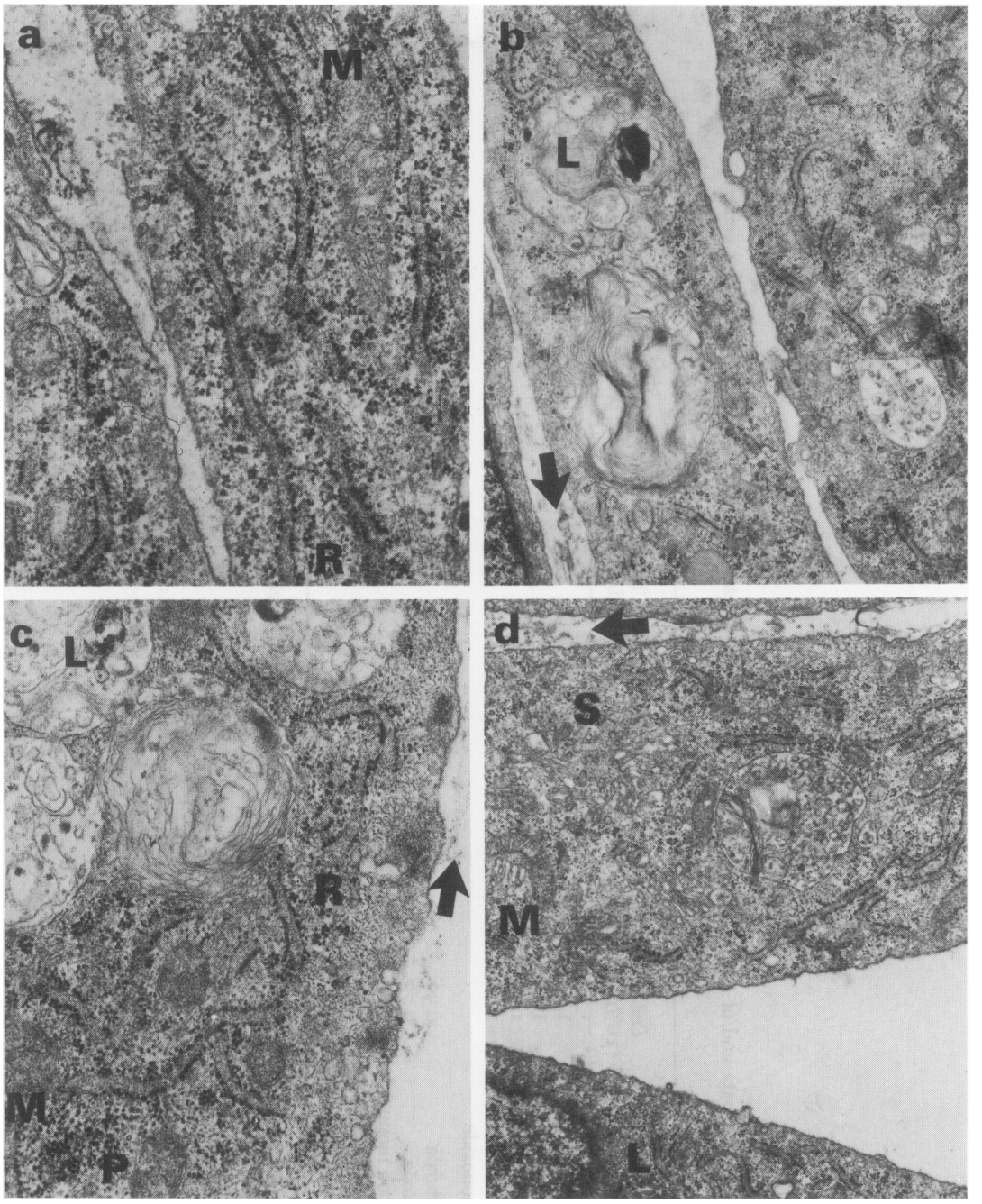

Fig. 2. Bovine endothelial cells. (*a*) Control (normoxic) bovine endothelial cells. Cytoplasm shows regular arrangement of RER (R), polysomes and mitochondria (M). × 27 000. (*b*) Hypoxic bovine endothelial cells. Hypoxia induces some cellular damage: reduction of the number of the RER cisternae, focal disorganization of mitochondrial membranes and lysosomes (L) filled by dense bodies and myelin figures. In some areas extracellular filamentous materials are visible (arrow). × 25 000. (*c*) Normoxic bovine endothelial cells plus carbon monoxide. Cytoplasmic organelles like polysomes (P), RER (R) and mitochondria (M) are well preserved. Some large lysosomes (L) filled with electron-dense material and myelin figures are well evident. Filamentous extracellular structures are associated with the plasma membranes (arrow). × 27 000. (*d*) Hypoxic bovine endothelial cells plus carbon monoxide. Cells show degenerative features: swelling of SER (S), and some lysosomes (L) with myelin figures, but mitochondria (M) and RER still have normal configuration. Extracellular granular material is present between cells (arrow). × 16 000.

Table 1. Effect of hypoxia and/or carbon monoxide on biochemical parameters in cultured porcine and bovine aortic endothelium

Species	Gaseous environment	Cell no. (10^6/plate)*	Protein (mg/10^6 cells)	Total protein (d/min/10^6 cells)	Total collagen (d/min/10^6 cells)	Total collagen d/min as % protein d/min/10^6 cells
Pig	Normoxia	1.87	0.19	1.6×10^6	151×10^3	9.5
	Hypoxia	1.41	0.27	1.6×10^6	107×10^3	6.6
	Carbon monoxide	1.68	0.25	1.5×10^6	87×10^3	6.0
	Hypoxia + carbon monoxide	1.06	0.39	2.3×10^6	173×10^3	7.8
Cow	Normoxia	0.92	0.39	1.4×10^6	158×10^3	10.9
	Hypoxia	0.79	0.49	0.7×10^6	117×10^3	16.6
	Carbon monoxide	0.87	0.49	1.5×10^6	150×10^3	10.3
	Hypoxia + carbon monoxide	0.89	0.35	1.4×10^6	330×10^3	23.9

Results shown represent one typical experiment out of three performed on each species.
* Results obtained from 3 plates.

method of Lowry *et al.* (1951) indicated that both hypoxia and carbon monoxide tended to increase the protein content of both the bovine and porcine cells, the one exception being that there is no effect on bovine cells treated with both hypoxia and carbon monoxide. (Table 1).

The use of pulse labelling with [^{3}H]-proline for 24 h showed however, that in bovine cells, hypoxia produced a large fall in total protein counts whereas it had no effect on porcine cells. Carbon monoxide alone produced very little change in either bovine or porcine cells; however when administered to hypoxic cells the pig showed considerable increase in total protein counts whereas no change was found in the cow (Table 1).

Effect of hypoxia and/or carbon monoxide on collagen synthesis per 10^6 cells

Pulse labelling with ^{3}H proline for 24 h confirmed that hypoxia depressed collagen synthesis per 10^6 cells in cow and pig. Carbon monoxide alone also produced a fall in the pig but not in the cow; however carbon monoxide plus hypoxia reversed the hypoxic effect in the pig and to a more dramatic extent in the cow as well.

Effect of hypoxia and/or carbon monoxide on collagen synthesis as a proportion of the total protein synthesized

Hypoxia increased the proportion of collagen synthesized in the cow, but diminished that in the pig. Carbon monoxide alone produced a similar effect in the pig but had no effect in the cow. When administered together carbon monoxide increased the proportion of collagen in the cow considerably, but to a much lesser extent in the pig (Table 1).

Effect of hypoxia and/or carbon monoxide on the collagen types in the cell layer and those released into the medium

As well as there being a species difference in the normal collagen profile the collagen types found in the endothelial cell layer normally differ in nature and relative proportions from those released into the medium (Levene *et al.* 1984). In this study we again found a selective retention of type V collagen

Table 2. Effect of hypoxia and/or carbon monoxide on the collagen types produced by cultured porcine and bovine aortic endothelial cells

Species	Gaseous Environment	Percentage collagen types in cell layer			Percentage collagen types in tissue culture medium		
		I	III	V	I	III	V
Pig	Normoxia	73	0	27	95	3	2
	Hypoxia	72	0	28	92	4	4
	Carbon monoxide	79	0	21	96	3	1
	Hypoxia + carbon monoxide	79	0	21	92	5	3
Cow	Normoxia	0	2	98	0	72	28
	Hypoxia	0	9	91	0	84	16
	Carbon monoxide	0	5	95	0	91	9
	Hypoxia + carbon monoxide	0	9	91	0	81	19

Results shown represent one typical experiment out of four performed on each species.

in the cell layers of both porcine and bovine endothelium. Treatment with either hypoxia and/or carbon monoxide had no effect on the collagen profiles in either cell layers or medium compared with normoxic controls (Table 2).

Discussion

We have examined the effects of two of the factors associated with cigarette smoking—hypoxia and carbon monoxide—on ultrastructural appearance and biochemical aspects of two species of cultured aortic endothelium—porcine and bovine—to see whether changes are produced and whether any such changes might provide insight into the aetiology of atherosclerosis. Our findings show that severe hypoxia depressed the cell numbers in both species but whereas carbon monoxide enhanced the fall in the pig, it counteracted the fall in the cow. Secondly, we found that both hypoxia and carbon monoxide generally increased the protein content of both cell species when measured by a chemical method; however the use of pulse labelling showed a fall in protein synthesis in the cow but not in the pig following 24-h hypoxia whilst carbon monoxide produced little change. One obvious possible explanation for these differences is that the chemical estimation of protein measures all the protein that has been synthesized since the cultures were first plated out, whereas the 24-h pulse label measures only the synthesis of protein during the particular 24 h during which the cells were subjected to the various gaseous environments.

Similar differences appear to occur in their effect on collagen synthesis following 24-h pulse labelling. As shown previously in cultures of pig aortic endothelial cells (Levene *et al.* 1982) and of human umbilical vein endothelium (Stavenow *et al.* 1983) hypoxia depressed collagen synthesis. Our findings now show that this occurs in the cow as well. Carbon monoxide, when given alone, also depressed collagen synthesis in the pig but had no effect in the cow. Moreover when the cells are subjected to carbon monoxide and hypoxia together, carbon monoxide tended to counteract the hypoxic effect.

Other differences emerged—in the cow, hypoxia increased the amount of collagen as a proportion of total protein synthesised, whereas in the pig that proportion diminished. Moreover the effect of carbon monoxide alone on this parameter differed in the cow from its effect in the pig.

The conclusions are firstly, that the effects of hypoxia and carbon monoxide vary depending on the cell species tested; secondly, that carbon monoxide appears in general to reverse the effects of hypoxia on most parameters measured. It is clear that hypoxia and carbon monoxide have differing actions and moreover act differently on differing species.

An exhaustive examination of the effect of these insults on the collagen phenotypes, produced by both species, showed that collagen polymosphism was unaffected. This is of interest as we now know that regulation of collagen polymorphism in this culture system is associated with such factors as occurence of sprouting (Levene *et al.* 1984), and duration of time in culture (Bartlet *et al.* 1984). This demonstrates that what was originally thought to be a highly variable parameter in a labile system is in fact regulated by certain factors, whilst remaining unaffected by others, such as hypoxia and carbon monoxide.

The relationship between collagen in the cell layer and that released into the medium shows a selective retention of type V collagen associated with the cells. This is of interest, as Madri *et al.* (1980) have shown by immunofluorescence that type V collagen caps the luminal surface of the endothelium in the aorta. In contrast to this we have shown (Carrington *et al.* 1984) that in 3T6 fibroblasts collagen types are found in the same proportions in the tissue culture medium and associated with the cell layers. This may prove to be a fundamental difference

between the properties of endothelium and the fibroblast.

We were surprised at the ability of our cells to survive such seemingly high concentrations of carbon monoxide in culture; however although carboxyhaemoglobin levels in smokers are usually found to be in the range of 4–8% Astrup *et al.* (1970) have shown that even after moderate smoking high concentrations of up to 20% carboxyhaemoglobin may occur in the blood.

The electron microscope evidence agrees generally with the biochemical data. Hypoxia was shown to cause loss of RER in both species whereas carbon monoxide seems to have had little effect. However apart from a small increase in cell death and some lysosomal damage, the synthetic apparatus does survive both hypoxia and carbon monoxide and can synthesize protein, including collagen. These findings are in accordance with those of Asmussen (1982) who has shown in ultrastructural studies in the human that umbilical arterial endothelium from smoking mothers, survives well but does show morphological changes even though the degree of hypoxia in smokers in the absence of respiratory failure is unlikely to be as acute as produced in the experiments reported here.

As well as cigarette smoking being a major risk factor in myocardial infarction, hypoxia and carbon monoxide, as two of the factors associated with cigarette smoking are known to affect the fetus during pregnancy often causing smoking mothers to have low-birthweight babies. The effect of heavy smoking by the mother is to cause chronic foetal hypoxia and there is evidence that hypoxia and carbon monoxide separately can have deleterious effects (Pirani 1978; Bewley 1984). In view of these species differences demonstrated here, it seems worthwhile exploring the response of human endothelium to such insults.

Acknowledgements

The authors are indebted to Dr Peter Royce of the Strangeways Research Laboratory, Cambridge, for the generous gift of the radiolabelled collagens I, III and V used in the fluorography; to Dr Mary Daniels of the Douglas Cancer Institute at the Clatterbridge Hospital, Liverpool, for the gift of the bovine endothelial cells used in this study; to Dr Tim Hunt of the Biochemistry Department, University of Cambridge for generously permitting the use of his scanning densitometry equipment; to Mr John Allen of the Histology Section of this department for his histological skills, and to Miss Wendy Gilson for electron microscopic and photographic assistance. This investigation has been supported by the Medical Research Council of whose External Staff C.I. Levene is a Permanent Member. Dr C. Fornieri is a visiting scientist from the Department of Pathology, University of Modena, Italy and is grateful to 'Legato Dino Ferrari', to EMBO and to the Hoechst Foundation for financial support.

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