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Research Article

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ABSTRACT Paired samples of an intact rabbit aortic intima-media preparation were incubated for short periods under aerobic or anoxic conditions in Krebs-bicarbonate buffer containing 6% albumin and 5 mM glucose. During aerobic incubation for as long as 1 h the preparation retained an electron microscopic (EM) appearance similar to that of tissue fixed *in situ*, and scanning EM confirmed the presence of an uninterrupted endothelial surface. After 2.5 min of anoxia there was widespread endothelial swelling, but the alterations in the EM appearance of these cells were not striking and did not progress during a subsequent 30 min aerobic incubation in fresh medium. After 10 min of anoxia there were marked and widespread alterations in endothelial cell structure, including loss of cell integrity, and numerous discrete interruptions in the endothelium were consistently observed on both transmission and scanning EM. After a subsequent 30 min aerobic incubation in fresh buffer, a major fraction of the luminal surface was denuded of endothelium. The aortic vascular smooth muscle cells did not exhibit evidence of irreversible anoxic injury after 2.5 or 10 min of anoxia or after subsequent aerobic incubation for 30 min. Exposure to anoxia for 10 min induced persistent alterations in the composite metabolism of the preparation during subsequent aerobic incubation in fresh medium; O_2 uptake was reduced, and the fraction of the glucose uptake that was accounted for by lactate production increased approximately 100%.

The observations suggest that aortic endothelial cells are dependent upon respiration for the preservation of normal ultrastructure and cell integrity, and probably derive the major fraction of their energy requirements from reactions linked to respiration. Under the conditions employed in these experiments, short periods of anoxia did not induce EM evidence of irreversible anoxic injury in aortic vascular smooth muscle cells; this negative result is not incompatible with other data suggesting that these cells normally derive the major fraction of their energy requirements from respiration. Aortic intima-media does not exhibit a high rate of aerobic glycolysis under aerobic conditions which preserve a normal EM appearance of the preparation, but this pattern of metabolism can be induced by prior anoxic exposure.

INTRODUCTION

Much of our present information on the metabolism of the arterial wall was derived from in vitro studies of isolated aortic intima-media preparations. Rabbit, rat, and swine aortic intima-media preparations have been reported to have a similar pattern of metabolism characterized by a high rate of aerobic glycolysis and a very low respiratory rate (1–8). This is the basis of the widely held view that the arterial wall normally derives the major fraction of its energy requirements from aerobic glycolysis. However, the presence of a high rate of aerobic glycolysis in an in vitro tissue preparation has frequently proven to be an artefact related to tissue injury or unphysiological incubation conditions (9). In a previous report, we noted that rabbit aortic intima-media prepared and

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incubated under conditions that preserve an intact endothelium and an electron microscopic (EM)[1] appearance similar to that of tissue fixed *in situ* does not exhibit a high rate of aerobic glycolysis and has a respiratory rate much higher than that previously observed in other aortic intima-media preparations (10). This suggested that the component cells might normally derive the major fraction of their energy requirements from reactions linked to respiration. The single cell layer of endothelial cells constitutes only a small fraction of the cellular mass of the aortic intima-media preparation; the bulk is comprised of vascular smooth muscle cells. Consequently, the pattern of metabolism in the small mass of aortic endothelial cells cannot be deduced from the composite metabolic activities exhibited by this preparation. We reasoned that if the aortic endothelial cells were dependent upon respiration for the provision of their energy requirements, this might be reflected by the development of marked alterations in their ultrastructure when the intact aortic intima-media preparation was incubated under anoxic conditions for a brief period. The studies that form the basis of this report demonstrate that the aortic endothelial cells are dependent upon respiration for the preservation of normal ultrastructure and cell integrity.

METHODS

Young, male, white, New Zealand rabbits (1.5–2.5 kg) were fed freely on Wayne rabbit ration (Allied Mills, Inc., Chicago, Ill.) plus carrots and lettuce. The rabbits were sedated with diazepam (2.0 mg/kg) i.m. 90 min before decapitation. The details of the preparation of paired pooled samples of intact rabbit aortic intima-media were previously described (10). In brief, the descending thoracic aortic was quickly removed and rinsed in a large volume of Krebs-Ringer bicarbonate buffer maintained at 37°C which contained 6% dialyzed bovine serum albumin and 5 mM glucose; the pH of the buffer was maintained at 7.4 by continuous gassing with 5% CO_2/95% O_2. The aorta was dissected free of adipose tissue and adventitia in fresh buffer of the same composition with several changes of buffer during the dissection, and the tissue cut into six equal segments which were pooled as previously described (10) to provide two samples from each aorta, each weighing approximately 100 mg. The samples were drained of medium on filter paper, quickly weighed on a torsion balance, and transferred to 25-ml Erlenmeyer flasks for incubation within 7–8 min of sacrifice; if the time required exceeded 10 min the samples were discarded. The flasks contained 5.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 6% dialyzed bovine serum albumin and 5 mM glucose pre-equilibrated with the appropriate gas phase at 37°C; for experiments carried out under the standard aerobic conditions the gas phase was 5% CO_2/95% O_2; for anoxic incubations the gas phase was 5% CO_2/95% N_2. Incubations were carried out for the times indicated in the text in a Dubnoff shaker set at 88 cpm. In specific experiments paired samples were first incubated for the same period under aerobic or anoxic conditions, and then transferred to fresh media of the same composition that had been pre-equilibrated with 5% CO_2/95% O_2 and incubated for an additional 30 min under aerobic conditions.

For examination by transmission and scanning electron microscopy, the tubular segments of aorta were quickly removed at the end of the incubation, drained of medium, and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature. Segments were then washed in 0.1 M phosphate buffer, pH 7.4, and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4. Entire segments were subsequently dehydrated in graded concentrations of ethanol through pure ethanol. Preparation of the tissue for thin section and scanning electron microscopy diverged at this point. For the preparation of thin sections, segments of aorta were transferred after dehydration in pure ethanol into propylene oxide and embedded in Epon 812 (Shell Chemical Corp., Div. Shell Oil Co., New York). Thin sections were cut with a diamond knife, mounted on coated grids, and examined with a Phillips EM 300 electron microscope (Phillips Instruments, Eindhoven, The Netherlands). For scanning electron microscopy the tubular segments of aorta were transferred from pure ethanol into pure acetone for 2 h. They were then cut into two longitudinal halves and processed through the critical drying point (11). Dried halves (endothelium upwards) were glued onto aluminum stubs, and then coated with gold in a sputtering cathode device. Specimens were examined with a Siemens-Autoscan scanning electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) at an accelerating voltage of 20 kV.

To fix the aorta *in situ*, rabbits were sedated and sacrificed as described above. The aorta was rapidly exposed and cannulated at the arch and just proximal to the bifurcation, and flushed with 50 ml of 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. A constant infusion of the same solution was then initiated through the proximal cannula at the rate of 40 ml/min at a pressure of 120 cm of water. The perfusion was continued for 30 min; the descending thoracic aorta was then excised, placed in fixative of the same composition, dissected free of adipose tissue, and cut into tubular segments. After fixation in fresh 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, the tissue samples were prepared for transmission and scanning EM as described above.

The possible progression or regression of the changes in the EM appearance of the aortic endothelium induced by 2.5 min of anoxia during a subsequent 30 min aerobic incubation was evaluated by a morphometric approach (12). (The progression of the alterations in the aortic endothelium induced by 10 min of anoxia during a subsequent 30 min aerobic incubation was so striking and consistent on both transmission and scanning EM examination that a qualitative evaluation was deemed adequate.) For the morphometric evaluation, paired samples from the same aorta were fixed immediately after a 2.5-min incubation under anoxic conditions and after a subsequent 30 min aerobic incubation; the tissue from four paired experiments was used for the analysis. Ultrathin sections were examined from three tissue blocks randomly selected from each of the two experimental conditions employed in each paired experiment; 18 electron micrographs, 6 from each section, were taken randomly at magnifications of both ×7,200 and ×26,900. The electron micrographs were recorded on

[1] *Abbreviations used in this paper*: EM, electron microscopic; Sv, surface area of the endothelium per unit volume of tissue; Vv, volume density.

70 mm film in a Zeiss EM 9 electron microscope (Carl Zeiss, Inc., New York). A carbon grating replica with 2,160 lines per mm was recorded on each film for calibration of the magnification, and the films were examined in a table projector unit.

The point counting method was used to estimate the volume occupied by endothelium per unit volume of aorta, or by endothelial cell mitochondria per unit volume of endothelium, i.e. the volume density (12). The volume density (Vv) of aortic vascular smooth muscle cells was estimated by the same method. A lattice of P_T test points was placed on the negative and the number of points enclosed within the profiles studies (P_i) was determined.

$$\text{Vv (volume density)} = P_i/P_T \qquad (1)$$

The volume density of mitochondria per unit volume of endothelium was determined in a similar manner from electron micrographs taken at the higher magnification. The endothelial cell mitochondria were classified as intact or swollen according to standard criteria, and the volume densities of the two groups of mitochondria computed independently; in addition, the number of intact or swollen mitochondria in the endothelium was determined and expressed as a percent of the total mitochondria observed.

The surface area of the endothelium per unit volume of tissue (Sv) was estimated by counting the number of intersections (I_L) of the surface profile per unit length of test lines of known length.

$$Sv = 2\ I_L \qquad (2)$$

Oxygen uptake was determined after timed aerobic or anoxic incubations by means of a model 53 biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio) and recorder; after the incubation the tissue was rapidly transferred to fresh medium of the same composition preequilibrated with 5% CO_2/95% O_2 at 37°C in a chamber of the oxygen monitor and its oxygen uptake continuously recorded. The methods for the determination of glucose uptake and lactate production were described previously (1). Tissue ATP and phosphocreatine concentrations were determined by the methods of Lowry et al. (13) in neutralized perchloric acid filtrates prepared from tissue samples rapidly frozen and powdered after incubation as described by Lowry et al. (13). The differences between the metabolic activities of paired tissue samples were analyzed for significance by the *t* test for paired comparisons, but the means and the standard error of the mean for groups were also computed (14).

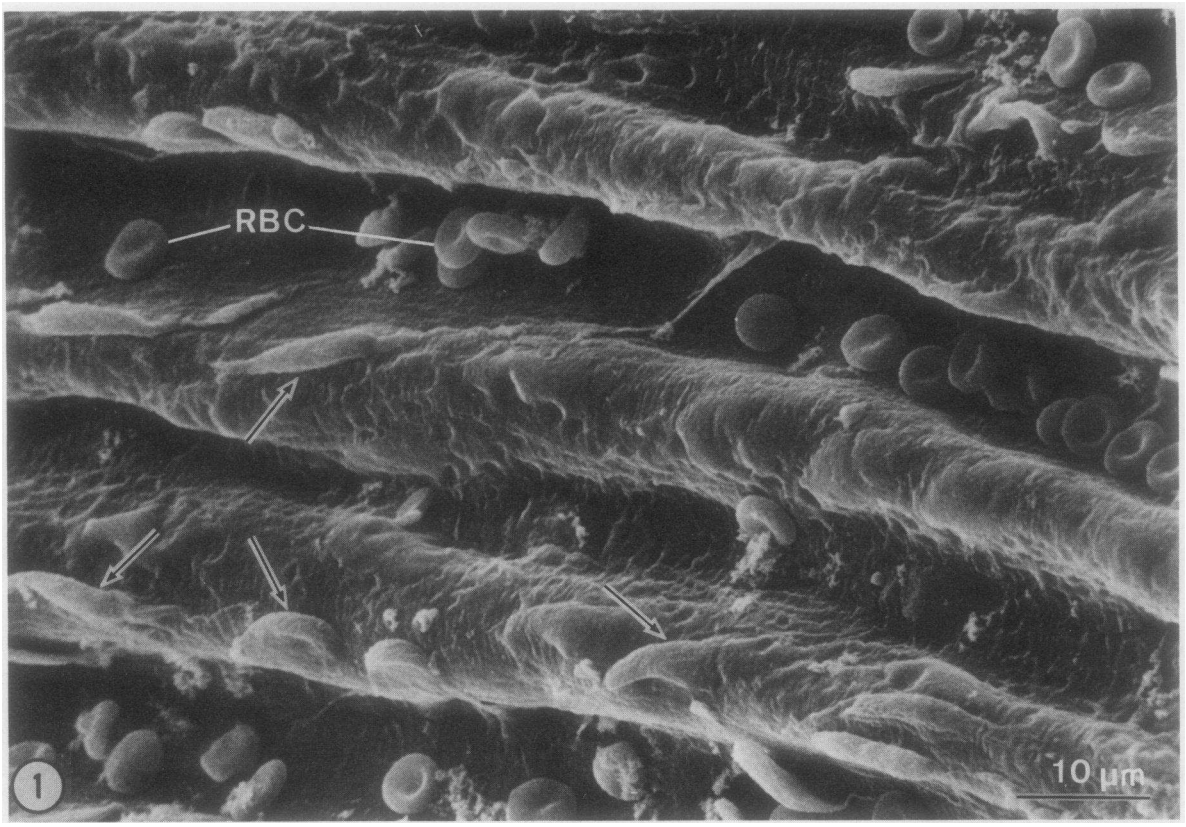
RESULTS

(*a*) The ultrastructure of paired samples of intact aortic intima-media was compared after incubation in the standard medium under aerobic or anoxic conditions for periods of 2.5 or 10 min (see Methods).

In all of the paired controls incubated under aerobic conditions for 2.5 or 10 min, the endothelial and vascular smooth muscle cells retained the normal transmission EM appearance found in tissue fixed *in situ*; this was anticipated, for, as previously reported, samples of the intact aortic intima-media preparation retain a normal EM appearance for at least 1 h under the standard incubation conditions with 5% CO_2/95% O_2 as the gas phase. The luminal surfaces of the paired controls incubated under aerobic conditions were also examined by scanning EM and compared first with that in tissue fixed *in situ* and then with the paired samples incubated under anoxic conditions. Fig. 1 illustrates the scanning EM appearance of the luminal surface of rabbit thoracic aorta fixed *in situ*; the endothelial surface is intact.[2] As illustrated in Fig. 2, the luminal surfaces of the paired controls incubated under aerobic conditions for 2.5 or 10 min consistently retained an intact endothelial surface. (In controls for other studies included in this report, the preservation of an intact endothelium on scanning EM examination was documented in samples incubated under aerobic conditions for periods as long as 1 h.)

In the paired samples incubated under anoxic conditions for 10 min (Fig. 4), there were striking and widespread alterations in the transmission EM appearance of the aortic endothelial cells as compared with those in paired controls incubated under aerobic conditions (Fig. 3). These alterations included loss of cell integrity and the consistent presence of areas in which the luminal surface was denuded of endothelium. On scanning EM examination, the luminal surfaces of samples exposed to anoxia for 10 min contained numerous discrete areas in which the continuity of the endothelium was interrupted (Fig. 5). In tissue samples exposed to anoxia for 2.5 min, there was widespread alterations in the transmission EM appearance of the aortic endothelial cells when compared with their controls; however, these were usually not striking and restricted to some degree of cell swelling and changes in the appearance of the mitochondria; they did not include loss of cell integrity. Thus anoxia induced alterations in the ultrastructure of aortic endothelial cells which progressed with increasing duration of anoxia, and which after

[2] In these studies scanning EM was used primarily to provide an additional assessment of the continuity of the endothelium. It is recognized that the scanning EM appearance of the aortic endothelial surface is modified by fixation without distension particularly with regard to the appearance of surface projections (15). In addition it has been reported that the regular longitudinal folds in the endothelial surface associated with undulations in the internal elastic lamina are eliminated when the distending pressure during fixation is maintained at physiological levels (15); in the reported studies pressures equal to or exceeding 80 mm Hg were used as an approximation of physiological diastolic pressure in adult rabbits. Hence the longitudinal folds observed in the endothelial surface of aortae fixed *in situ* at a perfusion pressure of 88 mm Hg for 30 min (Fig. 1), and in the short, isolated segments of aortic intima-media fixed of necessity without distension may not be present in the aorta under physiological conditions in vivo, but a comparison of these preparations with regard to evident discontinuities in the endothelium is valid. In each instance the samples of aortic intima-media exposed to anoxia were compared with paired controls from the same aorta fixed under the same conditions.


RBC
10 µm
1


10 µm
2

10 min reflected the loss of cell integrity in a significant population of endothelial cells.

The EM appearance of the vascular smooth muscle cells in tissue fixed immediately after 2.5 min of anoxia was essentially similar to that found in paired aerobic controls. After 10 min of anoxia the vascular smooth muscle cells still retained an EM appearance that did not differ markedly from that observed in paired aerobic controls with the exception of frequent alterations in their mitochondria (swelling, clarification of the matrix); there was no evidence of disruption of the plasma membrane, of densification of their nuclei, or of marked swelling of the cytoplasm or other organelles with the exception of the mitochondria.

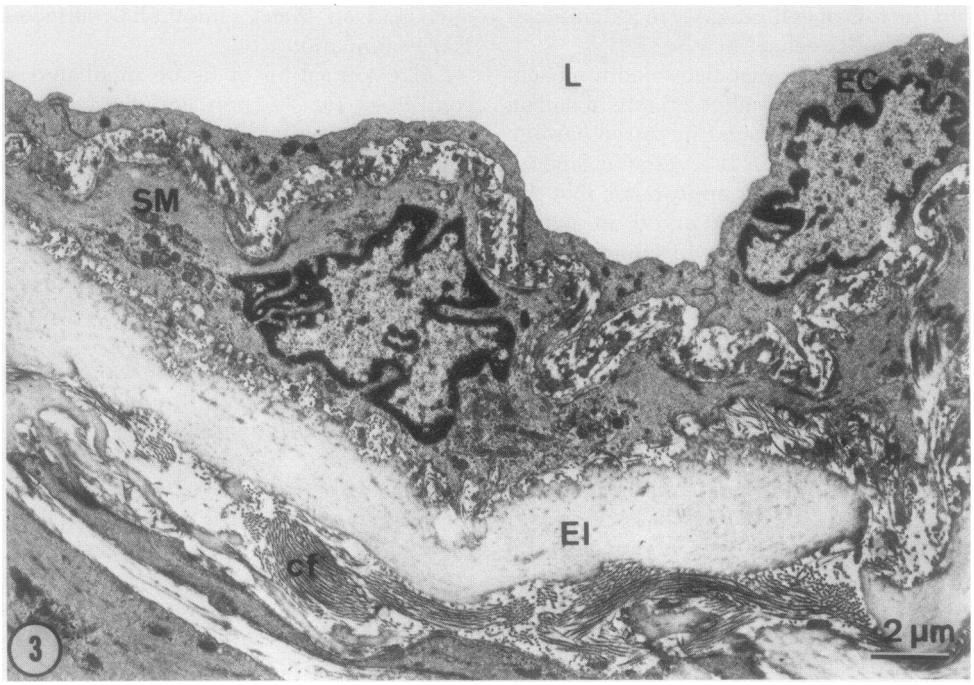
(*b*) A subsequent series of experiments was designed to examine the progression or regression of the EM alterations in aortic intima-media induced by exposure to anoxia when the tissue was transferred to an aerobic environment. Samples of intact aortic intima-media were incubated under anoxia conditions for 2.5 or 10 min, and then transferred to fresh incubation medium equilibrated with 5% CO_2/95% O_2 and incubated for an additional 30 min before they were fixed for EM examination. In the samples exposed to anoxia for 10 min and then incubated under aerobic conditions for 30 min, transmission EM examination indicated that much of the luminal surface was denuded of endothelium and that the remaining endothelial cells almost invariably had densified (i.e. pycnotic) nuclei. This interpretation was confirmed on scanning EM examination in which a major fraction of the luminal surface was consistently found to be denuded of endothelium, as illustrated in Fig. 6. When the scanning EM appearance of the samples fixed after a 10-min exposure to anoxia and a subsequent 30 min aerobic incubation (Fig. 6) was compared with that of samples fixed immediately after a 10-min exposure to anoxia (Fig. 5), it was apparent that despite the relief of the anoxia, there was a marked progression in the extent of the discontinuities in the endothelial surface during the ensuing 30 min. Thus exposure to anoxia for 10 min appears to induce acute, irreversible injury in a major fraction of the aortic endothelial cells. (Paired controls were subjected to two similar, consecutive incubations under aerobic conditions to evaluate possible alterations induced by tissue handling; these controls consistently retained an unaltered EM appearance and an intact endothelial surface on scanning EM examination.)

EM examination of tissue incubated under anoxic conditions for 2.5 min and then aerobically for 30 min occasionally demonstrated the presence of small discrete areas denuded of endothelium. However, in most instances the endothelial cell layer appeared to be intact; qualitatively there appeared to be some improvement in the EM appearance of the endothelial cells when compared with those in samples fixed immediately after 2.5 min of anoxia with regard to cell swelling and mitochondrial morphology. To permit a quantitative assessment of these observations, paired samples of tissue were prepared, and fixed immediately after a 2.5-min incubation under anoxic conditions and after a subsequent 30 min incubation under aerobic conditions, and subjected to morphometric analysis (see Methods). Although there was a tendency both for the surface area of the endothelium per unit volume of tissue and for the relative volume of endothelium to decline during a 30-min aerobic incubation after exposure to anoxia for 2.5 min, these changes were not statistically significant (Table I). However, the analysis did document a significant improvement in the morphology of the aortic endothelial cell mitochondria after the relief of anoxia of 2.5 min duration (Table I). In sum, while exposure to anoxia for 2.5 min induces widespread, albeit not striking alterations in the EM appearance of the aortic endothelial cells, longer periods of anoxia appear to be required to induce acute irreversible injury in a significant number of endothelial cells under the conditions employed in these studies.

The vascular smooth muscle cells in tissue exposed to anoxia for 2.5 or 10 min and then incubated for an additional 30 min did not exhibit EM evidence of irreversible injury such as disruption of the plasma membrane or nuclear pycnosis. The EM appearance of the smooth muscle cells in the tissue initially exposed to anoxia for 2.5 min and then incubated aerobically for 30 min was essentially similar to that observed in aerobic controls. The volume density of the vascular smooth muscle cells was estimated in paired samples fixed immediately after 2.5 min of anoxia and after a subsequent 30 min aerobic incubation (see Methods), but no significant difference in this parameter was observed (Table I). In tissue exposed to anoxia for 10

FIGURE 1 Normal rabbit thoracic aorta fixed *in situ*. This scanning electron micrograph shows the presence of an intact endothelium. The arrows point to elevations of the surface probably corresponding to the nuclear region of the endothelial cells.[2] RBC, erythrocytes. ×1,800.

FIGURE 2 Aortic preparation fixed after 10 min incubation under aerobic conditions. Scanning electron micrograph showing a continuous sheet of endothelial cells. ×2,300.



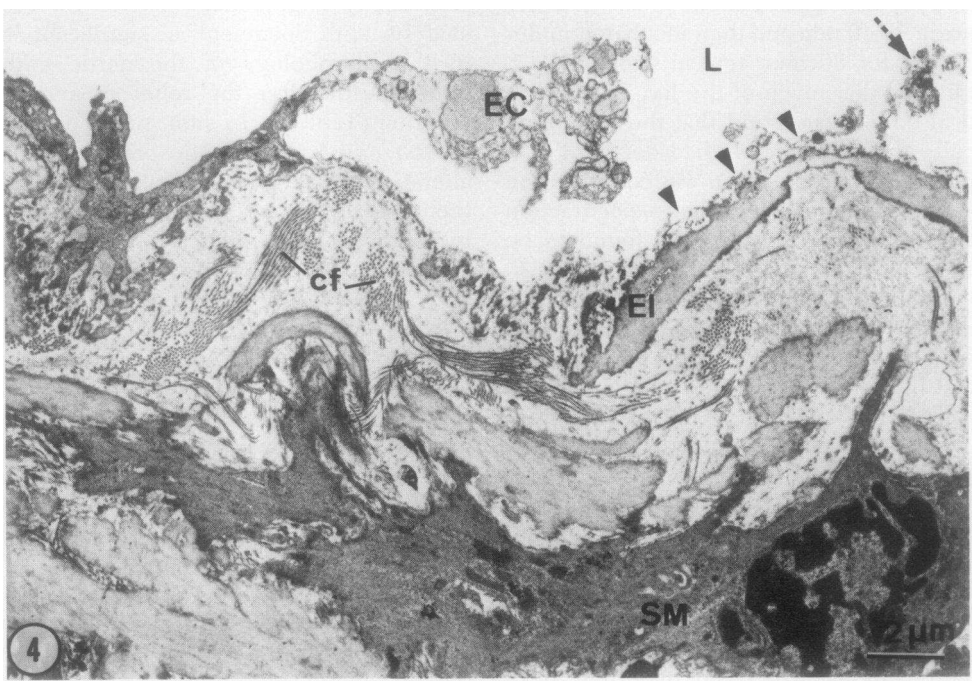


FIGURE 3 Aortic preparation fixed after 10 min incubation under aerobic conditions. The endothelium retains a normal ultrastructural appearance. L, aortic lumen; SM, smooth muscle; EI, elastica interna; cf, collagen fibers; EC, endothelial cell ×5,000.

FIGURE 4 Aortic preparation fixed after 10 min incubation under anoxic conditions. The dotted arrow points to fragments of necrotic endothelial cell. The arrowheads indicate areas in which the internal elastic membrane is exposed to the luminal surface. ×5,000.

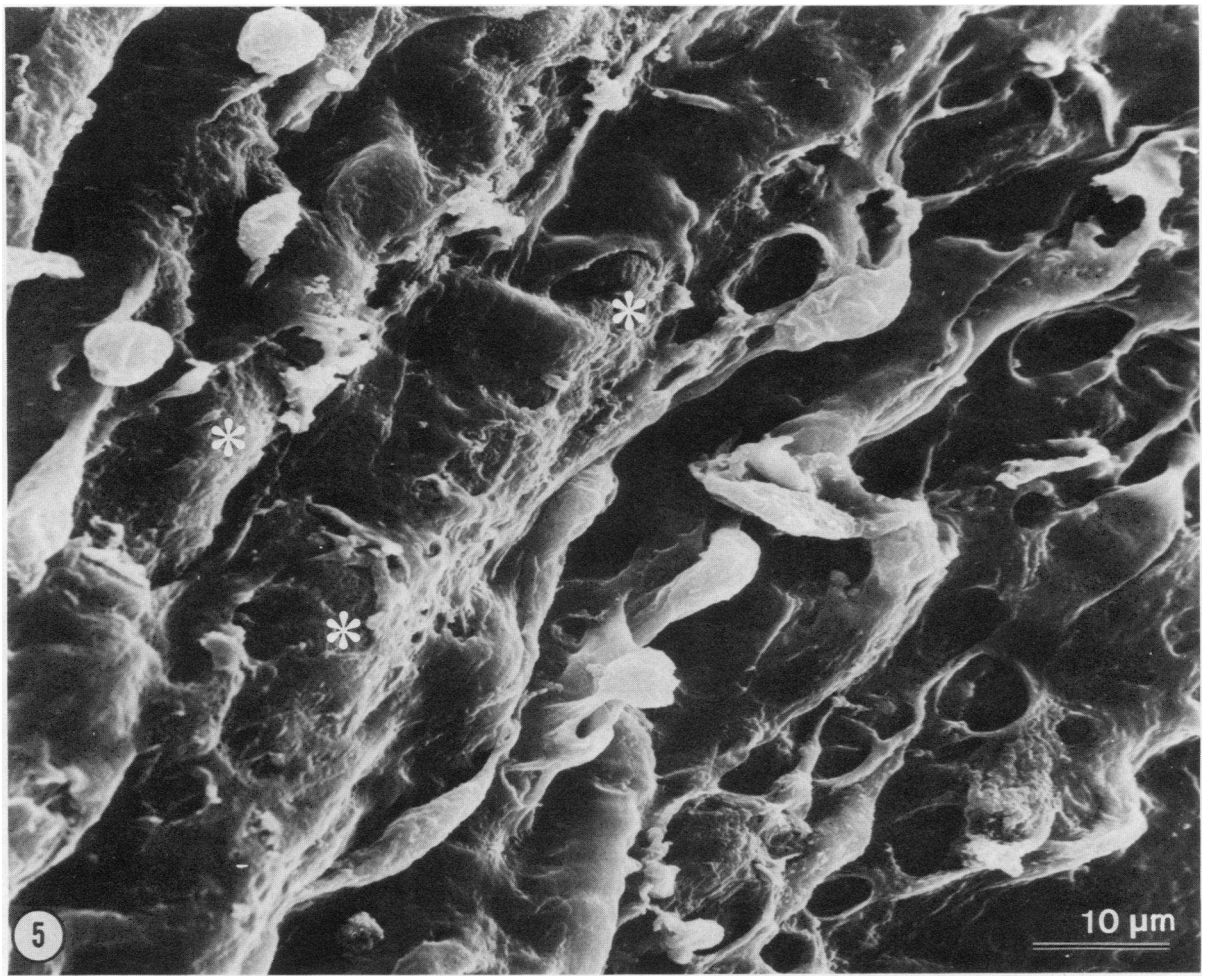


FIGURE 5 Aortic preparation fixed after 10 min incubation under anoxic conditions. This scanning electron micrograph shows a highly irregular endothelial surface. The asterisks indicate several areas where the endothelial sheet is interrupted. ×1,800.

min and then incubated aerobically for 30 min, there appeared to be a significant decrease in the frequency with which alterations in smooth muscle mitochondria were observed when compared with samples fixed immediately after 10 min of anoxia (*vide infra*), and the EM appearance of the smooth muscle cells did not differ markedly from that observed in aerobic controls. The EM appearance of the vascular smooth muscle mitochondria in paired samples fixed immediately after 10 min of anoxia, and after a subsequent 30 min aerobic incubation was assessed in a blind fashion in randomly selected electron micrographs from randomly selected blocks. This semiquantitative morphometric analysis confirmed the impression that the fraction of the vascular smooth muscle mitochondria exhibiting EM alterations after 10 min of anoxia decreased during a subsequent 30 min aerobic incubation. After 10 min of anoxia 35% of the mitochondria (339) found in the 53 vascular smooth muscle cells examined in this analysis showed significant alterations in their EM appearance. In samples fixed after a subsequent 30 min aerobic incubation, only 6% of the mitochondria (267) found in the 43 vascular smooth muscle cells included in the samples examined exhibited evidence of swelling or clarification of the matrix.

(*c*) To examine the possibility that exposure to anoxia for 2.5 or 10 min might induce persistent alterations in the composite metabolism of aortic intima-media, the O_2 uptakes of paired samples were compared under aerobic conditions in fresh medium after an initial incubation under aerobic or anoxic condi-

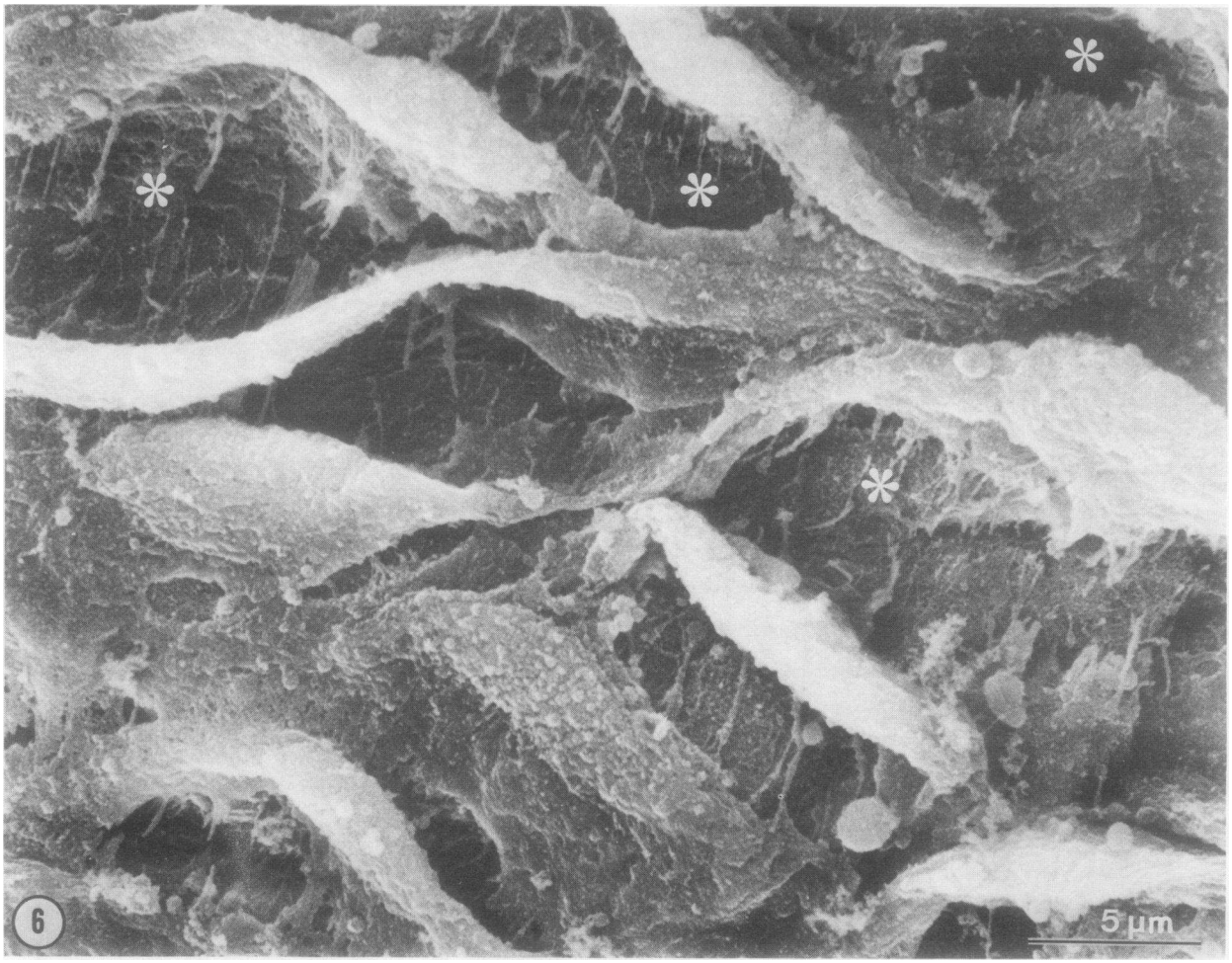


FIGURE 6 Aortic preparation fixed after 10 min incubation under anoxic conditions followed by 30 min incubation under aerobic conditions. Scanning electron micrograph showing a gross endothelial denudation in several areas (*). ×4,600.

tions. The O_2 uptake of samples examined immediately after a 2.5-min anoxic incubation was linear by the end of the equilibration period and was not significantly different from that of the paired aerobic controls (Table II). However, a comparison of the O_2 uptakes of paired samples examined immediately after 2.5 min of anoxia, and after a subsequent 30 min incubation under aerobic conditions, demonstrated a consistent and significant decline in respiratory rate in the 30-min after anoxia (Table II). It would appear that exposure to anoxia for 2.5 min is sufficient to induce persistent alterations in the respiration of the tissue. (The declining respiratory rate precluded meaningful estimates of the rates of glucose uptake and lactate production under these conditions.)

The O_2 uptake of samples examined immediately after exposure to anoxia for 10 min was linear by the end of a 5-min equilibration period, but was significantly lower than that of their paired aerobic controls (Table II). There was no significant difference in the O_2 uptake of paired samples examined immediately after 10 min of anoxia and at the end of a subsequent 30 min aerobic incubation (Table II). Thus exposure to anoxia for 10 min induces a persistently reduced but linear rate of respiration in aortic intima-media; subsequent control experiments demonstrated that this decreased rate of respiration remained linear for at least 1 h. The latter observation made it permissible to compare the rates of glucose uptake and lactate production by paired tissue samples during a 1-h incubation in fresh aerobic medium after an initial 10 min incubation under anoxic or aerobic conditions. The samples previously exposed to anoxia consistently demonstrated a decreased glucose uptake, an increased lactate production, and an increase of approximately 100% in the fraction of the glucose uptake that appeared to be accounted for by lactate production (Table II).

TABLE I

Morphometric Evaluation of Changes in the EM Appearance of the Aortic Endothelium during a 30-Min Aerobic Incubation after a 2.5-Min Anoxic Incubation

Incubation conditions	Anoxic 2.5 min	Anoxic 2.5 min + 30 min aerobic	*P*
Vv endothelium	0.0736±0.0052	0.0591±0.0077	NS
Sv endothelium	0.1753±0.0130	0.1549±0.0068	NS
Vv intact mitochondria	0.0144±0.0020	0.0229±0.0035	<0.05
Vv swollen mitochondria	0.0185±0.0028	0.0114±0.0034	NS
Percent intact mitochondria	51±2	73±8	<0.05
Ratio of intact/swollen mitochondria in endothelium	0.85±0.21	2.25±0.35	<0.001
Vv smooth muscle	0.2615±0.0075	0.2397±0.0183	NS

Paired samples of aortic intima-media were fixed for EM examination immediately after a 2.5-min incubation in the standard incubation medium with 5% CO_2/95% N_2 as the gas phase or after a subsequent 30 min incubation in fresh medium with 5% CO_2/95% O_2 as the gas phase; tissue from four paired experiments were examined by a morphometric approach (see Methods). Vv is the volume density of endothelium, endothelial mitochondria, or of smooth muscle, expressed as a fraction. Sv endothelium is the surface area of endothelium per unit volume of tissue expressed as a fraction. The values given in the table are the mean±SE for the two groups of tissues.

TABLE II

Effects of Prior Incubation Under Anoxic Conditions on Composite Metabolic Activities of Aortic Intima-Media during Aerobic Incubation

Conditions				
(*a*)	O_2 Uptake, *μl O_2/g wet wt/h*			
Aerobic controls	Post 2.5 min anoxia	Mean Δ±SE	*n*	*P*
200±4	200±4	0±2	(6)	NS
Post 2.5 min anoxia	Post 2.5 min anoxia + 30 min aerobiosis			
193±5	160±6	−33±6	(8)	<0.005
Aerobic controls	Post 10 min anoxia			
201±7	145±4	−55±4	(8)	<0.001
Post 10 min anoxia	Post 10 min anoxia + 30 min aerobiosis			
155±3	158±6	+3±5	(6)	NS
(*b*)				
Aerobic controls	Post 10 min anoxia	Mean Δ±SE	*n*	*P*
	Glucose uptake, *μmol/g wet wt/h*			
9.94±0.44	7.34±0.33	−2.59±0.36	(12)	<0.001
	Lactate production, *μmol/g wet wt/h*			
2.75±0.33	3.99±0.34	+1.24±0.21	(12)	<0.001
	Lactate production, *percent glucose uptake*			
14.3±1.6	29.0±2.4	+14.6±1.6	(12)	<0.001

(*a*) Oxygen uptake determined in fresh Krebs-Ringer bicarbonate buffer containing 6% bovine serum albumin and 5 mM glucose, pH 7.4, gas phase 5% CO_2/95% O_2, after timed incubations in the same medium under aerobic or anoxic conditions (i.e. gas phase 5% CO_2/95% N_2), and, where indicated, after transfer to fresh medium and incubation under aerobic conditions for an additional 30 min. (*b*) Activities observed during a 1-h incubation in fresh standard buffer under aerobic conditions by paired samples after an initial 10 min incubation under aerobic or anoxic conditions.

DISCUSSION

The premise on which these studies was based was that if a major fraction of the endothelial or vascular smooth muscle cells in intact aortic intima-media exhibited EM evidence of acute irreversible injury after short periods of anoxia under controlled in vitro conditions, this response would be inconsistent with the possibility that the specific cell type affected was normally adapted to derive the major fraction of its energy requirements from aerobic glycolysis. For in that situation, anoxic inhibition of respiration should not materially affect the normal pattern of energy metabolism, and would be unlikely to induce marked alterations in cell metabolism or composition that might contribute to a rapid loss of cell integrity. Thus the development of acute irreversible anoxic injury would support the view that the affected cell type normally derives a major fraction of its energy requirements from respiration. This approach was employed primarily to obtain evidence concerning the energy metabolism of the aortic endothelial cells which could not be derived from the composite metabolic activities of the aortic intima-media preparation because the smooth muscle cells constitute the bulk of its cellular mass.

Anoxia rapidly induced changes in the EM appearance of the endothelial cells in the intact aortic intima-media preparation under conditions in which an EM appearance similar to that observed in tissue fixed *in situ* is preserved in the presence of oxygen. These alterations progressed with increasing duration of anoxia. After 10 min of anoxia there was loss of cell integrity in a significant number of aortic endothelial cells, and a major fraction of these cells appeared to have suffered acute irreversible injury, as reflected by the rapid and extensive loss of endothelium that then ensued despite transfer to fresh medium and relief of the anoxic inhibition of respiration. These observations suggest that the aortic endothelial cells are dependent upon respiration for the preservation of normal ultrastructure and cell integrity; they are consistent with the conclusion that these cells derive the major fraction of their energy requirements from reactions linked to respiration. Since the aortic endothelial cells contain abundant mitochondria and are exposed to a stream of arterial plasma in life, this would appear to be a reasonable possibility. However, it has previously received inadequate consideration because of the belief that the components of the arterial wall derive the major fraction of their energy requirements from aerobic glycolysis and the lack of direct experimental support for this possibility. The controlled conditions employed in these experiments permit a more convincing attribution of the structural alterations in the aortic endothelial cells to the effects of anoxia than is possible in experiments in which a vessel is ligated or the animal rendered anoxic. A host of factors that might operate in vivo to modify or accelerate the effects of anoxia on the aortic endothelial cells (e.g. erythrocytes, platelets) are absent under the conditions employed in these studies. Consequently, although a period of anoxia longer than 2.5 min was required to induce irreversible injury in a significant number of aortic endothelial cells, this observation cannot be extrapolated to assess the possible susceptibility of aortic endothelial cells to acute anoxic injury under conditions that might be encountered in life. These observations should provide an impetus to studies to determine the manner in which hypoxia may affect arterial endothelial structure, its interaction with plasma constituents, and its presumed function in regulating access of specific plasma constituents to the subintimal space. Alterations in the EM appearance of aortic endothelium have been reported in rabbits exposed to chronic hypoxia (16) or carbon monoxide (17); however, the extent to which these alterations directly reflect effects on endothelial cell respiration remains to be clarified. The extent to which endothelial cells in other regions of the vascular system resemble or differ from aortic endothelial cells in their dependence upon respiration requires examination, as does the question of whether this pattern of metabolism is retained in aortic endothelial cells adapted to growth in tissue culture.

Since the smooth muscle cells constitute the bulk of the cellular mass of the intact aortic intima-media preparation, it is reasonable to assume that they make the preponderant contribution to the composite metabolic activities observed when this preparation is incubated under the standard aerobic conditions. The relatively high oxygen uptake of the preparation under these conditions and the very small fraction of the glucose uptake that can be accounted for by lactate production suggest that the vascular smooth muscle cells normally derive the major fraction of their energy requirements from respiration. This conclusion is not incompatible with our failure to demonstrate EM evidence of irreversible injury in smooth muscle cells in tissue exposed to anoxia for 10 min or after subsequent aerobic incubation for 30 min. For, a negative result with the experimental approach employed in these short term studies does not permit firm conclusions. The inconclusive nature of a negative result stems from the fact that cells that normally derive the major fraction of their energy requirements from respiration may be capable of withstanding varying periods of anoxia under specific experimental conditions without sustaining irreversible injury. This presumably reflects, among other factors, a varying capacity to provide their energy require-

ments from an accelerated rate of glycolysis, and to withstand the expected alterations in their redox state and chemical composition without the loss of cell integrity. A marked Pasteur effect has been observed in the intact aortic intima-media preparation (10), and a comparison of the concentrations of ATP and phosphocreatine in paired aerobic controls and samples exposed to anoxia for 10 min failed to demonstrate any significant differences. (In six paired experiments the mean Δ in ATP concentrations in samples exposed to anoxia for 10 min and in paired aerobic controls was -50 ± 26 nmol/g wet wt, P = NS, and the mean Δ in phosphocreatine concentrations was -30 ± 31 nmol/g wet wt, P = NS). These observations suggest that during a 10-min period of anoxia accelerated glycolysis prevents a marked decrease in ATP and phosphocreatine concentrations in the smooth muscle cells. However, these observations clearly do not exclude the possibility that the structure or metabolism of the aortic vascular smooth muscle cells may not be subject to adverse modification by anoxia or hypoxia under other experimental conditions or in life. It must be noted that only relatively short periods of observation were possible in these in vitro studies, and that the energy requirements of the vascular smooth muscle cells may be reduced in tubular segments of aortic intima-media by the absence of the stretching forces to which they are exposed in life.

Even very short exposure to anoxia (2.5 min) induces persisting alterations in the metabolism of aortic intima-media during subsequent aerobic incubation, as reflected by a declining respiratory rate. This occurs in the face of improvement in the EM appearance of the endothelial cell mitochondria, and in the absence of significant changes in the appearance of the smooth muscle cells or in their estimated volume density. While the origin of this effect remains unclear, it is an obvious potential source of artefacts in studies of the metabolism of this tissue.

These studies provide additional support for the view that the high rates of aerobic glycolysis and lower respiratory rates observed in other aortic intima-media preparations are probably artefacts. This pattern of metabolism was not present in aerobic controls, but was reproducibly observed in aortic intima-media exposed to anoxia for 10 min before incubation in fresh aerobic medium. Despite their normal EM appearance, the elevated rate of aerobic glycolysis exhibited by the surviving smooth muscle cells undoubtedly reflects alterations resulting from anoxic injury to the tissue.

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