Effects of Oxygen and Glucose Deprivation on Vasoactivity in Isolated Bovine Middle Cerebral Arteries

PHILLIP E. VINALL, AND FREDERICK A. SIMEONE

SUMMARY The effects of oxygen and glucose deprivation on vasoactivity were investigated using helical strips of bovine middle cerebral artery. Hypoxia, created by reducing the PO2 of the bath, or oxidative inhibition with 2,4 dinitrophenol (DNP) or sodium azide, significantly reduced contractions induced by serotonin. Normal tonic contractions induced with fresh and aged whole blood, or 5-HT became phasic and quickly relaxed to baseline in a hypoxic environment.

Glucose elimination from the Krebs medium, or the inhibition of the glycolytic pathway with iodoacetic acid (IAA), did not significantly reduce serotonin-induced contractions. However, contractions were inhibited more with the combination of oxygen and glucose deprivation, or DNP + IAA, than with oxygen deprivation alone. Efforts to produce rigor in this preparation by oxygen/substrate reduction or metabolic inhibition were unsuccessful.

Tonic contractions induced by 70 mM potassium became phasic as the Ca++ concentration was reduced. Contractions resulting from the readdition of Ca++ to arteries exposed to calcium-free high potassium solution were significantly reduced in the presence of oxidative and/or glycolytic inhibitors. The uptake of 45Ca++, as measured by the lanthanum technique, decreased as the bath PO2 was reduced in both serotonin stimulated and unstimulated arteries. Glucose deprivation alone did not affect 45Ca++ uptake.

This study suggests that hypoxia has a direct inhibitory affect on cerebral vasoactivity mediated by reductions in saraplastic Ca++ uptake.

Methods

Tissue Preparation

Bovine middle cerebral arteries (MCA) were obtained from a local slaughterhouse, cut into helical strips and suspended isotonically from Harvard transducers in modified Krebs solution kept at 37°C. The mM composition of the Krebs solution was as follows: NaCl, 118.9; KCl, 4.7; KHPO4, 1.2; MgSO4, 1.2; CaCl2, 1.2; NaHCO3, 14.9; dextrose, 11.2. The control gas mixture contained 12% oxygen, 5% carbon dioxide, balance nitrogen. The CO2 concentration was always maintained at 5%. Oxygen (95%, 12%, 0%) and nitrogen (0%, 83%, 95%) concentrations were combined to produce varying gas mixtures. These oxygen concentrations produced the following bath PO2 (mm Hg): 95%O2 = 500-600, 12%O2 = 90-100, 0%O2 = 10-25. The osmolarity of all solutions was 275 and pH was 7.4. Arteries were allowed to equilibrate for 60 minutes in Krebs solution with 1.25 grams tension applied. Viability of the vessel was determined by the rate of adjustment of the strips to resting length upon the application of tension. Strips that did not stretch at a rate of 0.5 mm/min were rejected as too damaged. Contractions were expressed in mm/cm resting length of the strip (RLo).

Solutions and Drugs

The lanthanum-HEPES solution contained the following (mM): NaCl, 122; KCl, 5.9; MgCl2, 1.2; dextrose, 11.2; CaCl2, 10; HEPE-Tris, 6. The pH of the HEPE-Tris stock solution was adjusted to 7.4 with NaOH. Fresh rabbit and human whole blood were extracted and added to the bath before clotting occurred. Aged whole blood was produced by placing human and rabbit blood in an incubator at 37°C for seven days. Cumulative calcium contractions were induced in arteries washed with calcium-free 70 mM KCl solu-
tion. High K⁺ solution was made by increasing the K⁺ concentration and decreasing the Na⁺ to maintain an osmolarity of 275 milliosmoles. Metabolic inhibition in high potassium solution was produced by removal of glucose and oxygen from the bath medium according to the method used by Bose & Bose⁹ to induce rigor in taenia coli.

PO₂ of the bath was measured with an IL pH/gas analyzer, Model 113. Serotonin hydrochloride (5-HT), sodium azide (NaN₃), 2,4 dinitrophenol (DNP), and sodium iodoacetic acid (IAA) were obtained from Sigma Chemical Co. All drugs were dissolved in double deionized water. Concentrations are given as the salt and expressed as the final molar concentration in the bath.

**Calcium Uptake**

⁴⁵Ca²⁺ uptake was measured with the lanthanum technique. This method is based on the principle that 10 mM lanthanum displaces extracellular calcium, blocks both Ca²⁺ uptake and efflux, and does not enter the cell to alter cellular Ca²⁺ distribution. Cerebral arteries were placed in Krebs solution for 60 min before the tissue was labelled with ⁴⁵Ca (0.05μCi/ml). Radioactive ⁴⁵Ca⁺⁺ was obtained as CaCl₂ from New England Nuclear Co. The labelled arteries were allowed to equilibrate for 30 min before 10⁻³ M 5-HT was added to the bath. Arteries were left in contact with 5-HT or the control medium for one hour before being washed four times with glucose-free Krebs solution containing ⁴⁵Ca⁺⁺ with the same specific activity as before, then with a non-radioactive lanthanum-HEPES solution. The strips were left in the latter solution for one hour. After blotting and weighing, the arterties were dried overnight and dissolved in 1 ml Protosol (New England Nuclear Co.) at 60°C for three hours. Standard liquid scintillation counting, with appropriate controls, was performed and the efficiency was determined with internal standards. Calcium content was expressed as mM/kg wet weight of tissue.

**Statistical Treatment of Data**

Statistical analysis of calcium uptake values and dose response contractions were performed with the Student's t-test using a paired test for correlated variables. Differences were considered significant at the 95% confidence level. Mean ± SEM are reported for all uptake and contractile values.

**Results**

Bovine MCA were subjected to 95% and 0% oxygen aeration for 30 min before being contracted with fresh or aged rabbit, or human whole blood (0.1ml), or 5-HT (10⁻⁶ M). Normal tonic contractions that can last up to 6 hours in this preparation became phasic in the low oxygen environment and relaxed to baseline (fig. 1). Similar phasic responses also occurred with aged blood in the hypoxic bath.

Serotonin responses were significantly (p < .01) depressed in the absence of oxygen (fig. 2), while the absence of only glucose did not significantly affect similar contractions. Arteries were washed four times with glucose-free Krebs solution for 30 min. before 5-HT contractions were induced. The combination of 0% oxygen and glucose-free washings reduced the 5-HT-induced contractions more than oxygen deprivation alone. Serotonin responses returned to control values upon the readdition of glucose and oxygen after four hours of no oxygen or glucose.

A similar depression of 5-HT responses occurred when the arteries were exposed to the oxidative inhibitors DNP (10⁻⁴ M) or NaN₃ (10⁻⁵ M), or the glycolytic inhibitor IAA (2 × 10⁻⁵ M) for 30 min prior to stimulation (fig. 3). NaN₃ inhibited the 5-HT responses throughout the dose response range, while DNP inhibited contractions induced by 10⁻⁵ and 10⁻⁶ M 5-HT only. Significant (p < .01) reductions in the amount of contraction induced by 5-HT occurred in the presence of DNP and NaN₃, but not IAA, while the combination of DNP and IAA totally eliminated all contractile responses. DNP and glucose-free Krebs washings also completely inhibited all 5-HT responses. Responses to 5-HT returned to control values after washing the vessels with Krebs without metabolic inhibitors.

When MCA were exposed to 70 mM potassium solution containing 1.2 mM Ca²⁺, a sustained contraction occurred. As the Ca²⁺ concentration was reduced, the K⁺ response became phasic (fig. 4). If all calcium was removed from the 70 mM K⁺ solution, the K⁺ response quickly relaxed almost to baseline within 20 min. If Ca²⁺ was readded to the above calcium-free, high K⁺ solution, contractions again oc-
Figure 2. Effects of 0% oxygen and glucose-free Krebs washings on serotonin cumulative dose responses in MCA. Each point is the mean ± SEM. Points below (*) are significantly (p < .01) different from their corresponding control (12% oxygen/glucose) responses. Rlo is optimal resting length of the strip.

occurred which were concentration dependent (fig. 5). Most Ca++-induced contractions in the presence of 70 mM K+ were equally and significantly (p < .01) depressed by the presence of DNP (10^-4 M), NaN3 (10^-5 M) or IAA (2 × 10^-5 M). Inhibitors were added 30 min. before Ca++ was readded. Like the 5-HT-induced contractions, the combination of DNP and IAA produced complete inhibition of all Ca++ responses.

45Ca++ uptake studies revealed a relationship between the availability of oxygen and the amount of intracellular calcium (fig. 6). 45Ca++ was measured in the presence (stimulated) and absence of 10^-3 M 5-HT by the lanthanum technique. When bath oxygen concentration was decreased from 95% to 12%, the mean 45Ca++ uptake decreased from 0.227 ± .02 to 0.183 ± .01 mM/Kg wet wt. in unstimulated arteries (p <

Figure 3. Effects of oxidative (DNP, 2,4 dinitrophenol; NaN3-sodium azide) and glycolytic (IAA-iodoacetic acid) inhibitors on serotonin cumulative dose responses. Each point is the mean ± SEM. Points below (*) are significantly (p < .01) different from their corresponding control (12% oxygen/glucose) responses.

Figure 4. Effects of Ca++ reduction and elimination on tonicity of 70 mM K+ contraction in MCA. Artery was washed with potassium (K+) solution at arrows.

Figure 5. Effects of oxidative (DNP, 2,4 dinitrophenol; NaN3-sodium azide) and glycolytic (IAA-iodoacetic acid) inhibition on cumulative Ca++ dose responses in MCA. Ca++ was added to calcium-free 70 mM K+ solution 30 min after the addition of the metabolic inhibitors. Each point is the mean ± SEM. Points below (*) are significantly (p < .01) different from their corresponding controls (no inhibitors, 12% oxygen/glucose) responses.
METABOLIC INHIBITION OF MCA VASOACTIVITY/Vinall & Simeone

Discussion

This study indicates that hypoxia has a direct inhibitory affect on cerebral vasoactivity that is associated with a reduction in the uptake of Ca++ into the sarco-

plasma. Whole blood- and serotonin-induced contractions were reduced in hypoxic and metabolically inhibited environments. The tonic part of the contraction appeared more sensitive to hypoxia than initial quick phasic part. 45Ca++ uptake was significantly reduced when the bath PO2 was reduced.

Hypoxia can induce a contraction in isolated pulmonary arteries,14, 15 aorta,16 and in at least one study, hypoxic induced contractions in the presence of 5-HT have been demonstrated in the dog basilar artery.17 On occasion we also noted small hypoxia-induced contractions in the presence of 5-HT that quickly relaxed to baseline, but this response was not consistent from artery to artery. This could be due to our inability to reduce bath PO2s to low enough levels to consistently observe this phenomenon. Even in the presence of 0% oxygen, bath PO2s as high 25 mm Hg were sometimes detected. In the taenia coli intracellular Ca++ increases during hypoxia18, 19 due to an increase in extracellular calcium influx, while a decrease in the sequestration of Ca++ ions in binding stores occurs in airway smooth muscle.20 However most studies indicate that hypoxia induces a reduction of vascular activity21-22 and this reduced vasoactivity may be associated with reduced 45Ca++ uptake.23

In excitation-contraction coupling in smooth muscle calcium can be obtained from either extracellular sources or released from cellular storage sites. However, cerebral arteries appear to be more dependent on extracellular than on sequestered cellular calcium for contractile responses than other arteries.14, 24 The initial fast part of the contraction appears regulated by a Ca++ influx process different from that controlling the slow tonic part of the contraction.25 Contractions activated by K+ and sensitive to D600, and those stimulated by alpha-adrenoceptor activation and relatively resistant to D600 are associated with two separate Ca++ entry pathways, a potential and receptor operated channel, respectively.25, 26 In this study hypoxia affected the tonic part of the 5-HT or whole blood contraction more than the phasic, and also significantly reduced the Ca++-induced contraction in 70 mM K+ solution. The reduction of bath Ca++ decreased the steady tonic response normally produced by high K+, more than the initial fast part of the contraction. The fact that a contraction occurred in the absence of calcium probably indicates that small amounts of Ca++ may remain in the extracellular spaces. If arteries are pre-washed with Ca-free Krebs containing 1.0 mM EGTA, no contraction results when the arteries are stimulated with 70 mM K+ calcium-free solution (unpublished observation). Thus, hypoxia may affect the direct influx of extracellular Ca++, more than the release of sequestered Ca++ (either intracellular or membrane bound) associated with the potential and receptor operated calcium channels.

Contractions induced by 5-HT were affected more by the lack of oxygen or the presence of oxidative inhibitors, than by the lack of glucose or the presence of a glycolytic inhibitor. 45Ca++ uptake was also not affected by the absence of glucose. However, arteries

Figure 6. Effects of 95% (PO2 @ 550 mm Hg), 12% (PO2 @ 95 mm Hg), 0% (PO2 @ 18 mm Hg) oxygen and the absence of glucose on 45Ca++ uptake in stimulated (5-HT) and unstimulated (control) MCA. Absolute values ± SEM of 45Ca++ above bars. Significant differences in the amount of 45Ca++ uptake between control and 5-HT treated arteries are indicated by (*) p < .001, (**) p < .01.
continued to contract even in near anoxic environments when glucose was present. The absence of oxygen and glucose, or the presence of both a glycolytic and oxidative inhibitor reduced the 5-HT responses more than the lack of oxygen alone. Other studies have reported total loss of vascular contractility in the absence of both oxygen and glucose. In this study significant, though reduced, contractions occurred in the absence of both oxygen and glucose. This may indicate a role for glycolysis in the contractile process in this artery and/or the presence of large stores of glycogen. However smooth muscle cells are known to contain small stores of glycogen. A more probable explanation for the relatively large 5-HT response may be that 0% oxygen does not lower bath oxygen below levels that totally inhibit contraction in this vessel. Even after three hours of 0% oxygen aeration PO2 levels as high as 25 mm Hg could still be detected in the bath on occasion. This may be sufficient oxygen to support the contractions seen in the absence of oxygen and glucose. These same contractions were totally eliminated when both the oxidative and glycolytic pathways were poisoned with the combination of DNP + IAA. The finding that DNP + glucose-free treatment produced complete loss of vasoactivity also argues for the existence of small glycogen stores in this artery.

The decrease in Ca2+ uptake and contractility due to hypoxia may be related to the availability of ATP. In taenia coli hypoxia is associated with decreased ATP levels. Similarly, ATP decreases during ischemia in human cerebral tissue. Oxidative phosphorylation provides most of the energy required to synthesize ATP directly during contraction of vascular smooth muscle, and it was the oxidative mechanism which appeared more sensitive to hypoxia in this preparation. Unlike skeletal muscle, smooth muscle contains smaller energy stores of ATP, creatine phosphate and glycogen. Thus in vivo, cerebral arteries may be dependent upon adequate supplies of oxygen and ATP to offset pathological states which tend to reduce blood flow, such as long term vasospasm.

Efforts to demonstrate the existence of rigor in this preparation were disappointing. Bose & Bose, and later Butler et al, were able to induce rigor in other smooth muscle in the absence of glucose and oxygen. Rigor is associated with low ATP levels, increased resistance to stretch and occurs independent of elevations of cytoplasmic calcium. An explanation of long-term vasospasm following subarachnoid hemorrhage in terms of rigor is an attractive hypothesis. The secondary ischemia due to reduced blood flow may reinforce the initial blood induced contraction by reducing the ATP stores required to drive calcium back into the sarcoplasmic reticulum, mitochondria and extracellular space, and hence foster relaxation. Again our inability to demonstrate a rigor type response in this artery may reflect our inability to reduce both oxygen levels, hence ATP levels, to the critical level required to induce a rigor type contraction. Even in the absence of both glucose and oxygen, significant 5-HT contractions still occurred in this preparation indicating adequate energy was available for vasoconstriction. Carotid arteries stimulated with high K+ solution containing metabolic inhibitors do not relax when the vessels are returned to a physiological environment. This condition coincides with the almost complete loss of ATP and phosphorylcreatin. Thus, in the presence of an extreme hypoxic environment and the significant loss of smooth muscle ATP, arteries may remain contracted, even in the absence of the original stimulus.

In conclusion, we feel the reduced vasoactivity seen in response to hypoxia in the cerebral vascular system is the end result of reduced calcium uptake into the smooth muscle cell. Cerebral arterial contractions were depressed by decreasing oxygen availability, and these reduced responses were reflected by reduced amounts of 45Ca2+ uptake.

Since 45Ca2+ uptake seems to be directly related to oxygen availability, and 10–20% oxygen can produce physiological bath PO2, we question the use of such unphysiological oxygen concentrations as 95% O2 used in most in vitro smooth muscle experiments, particularly in studies which use thin wall vessels like the bovine cerebral artery. Such high concentrations of oxygen may in fact favor unphysiological amounts of calcium entering the cell, or as documented in porcine coronary arteries suppress local regulation of arterial tone.

References

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Stroke. 1986;17:970-975
doi: 10.1161/01.STR.17.5.970

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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