Hypoglycemic Hyperemia in Retina of Newborn Pigs
Involvement of Adenosine

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**Purpose.** To determine whether retinal blood flow increases in response to perinatal hypoglycemia and whether the vasodilator adenosine is involved in mediating the hyperemic response.

**Methods.** Retinal fluorescein videoangiography was undertaken in intact eyes of isoflurane-anesthetized piglets using intracarotid injections of sodium fluorescein. Angiograms were recorded to videotape and analyzed off-line by image analysis software to determine stimulus-induced changes in mean arteriovenous transit times, and arteriolar and venular diameters, from which retinal blood flow was calculated. Two groups of animals were rendered hypoglycemic (blood glucose = 19 ± 1 mg/dl) by insulin (25 IU/kg, intravenously), and angiograms were obtained at 10-minute intervals for 0.5 hour of hypoglycemia. One group (n = 5) served as controls. In the other (n = 5), the nonspecific adenosine receptor antagonist 8-phenyltheophylline (8PT) was administered intravenously approximately 15 minutes before hypoglycemia to examine the role of adenosine in the hemodynamic response to hypoglycemia.

**Results.** Acute hypoglycemia was associated with an increase in mean retinal blood flow of 94 ± 18% (P < 0.002). However, in animals pretreated with 8PT, this hyperemic response was severely attenuated, primarily by an effect on arteriovenous transit time. In these latter animals, mean retinal blood flow only increased 19 ± 10% in response to hypoglycemia (P = 0.13 versus normoglycemic baseline; P = 0.007 versus untreated hypoglycemic animals). All other hemodynamic variables were similar between animal groups.

**Conclusions.** Acute hypoglycemia causes a compensatory increase in retinal blood flow in the perinatal period. Because the adenosine receptor antagonist 8PT attenuated this hyperemic response, it is concluded that adenosine is involved in eliciting the increase in retinal blood flow that accompanies hypoglycemia. Invest Ophthal Vis Sci. 1996; 37:86–92.

Hypoglycemia is encountered often in newborns,1,2 and the resultant injury of central nervous system tissue may impair neurologic development.3 Although studies of the effects of hyperglycemia on retinal vascular hemodynamics in adults with diabetes are widespread,4–5 only one investigation of the retinal vascular response to hypoglycemia has been reported to date6; in this adult animal model, hypoglycemia was associated with an increase in blood velocity in large veins. Whether retinal blood flow increases in response to reductions in glucose supply during the perinatal period is unknown. Neurons in the perinatal period may rely on the metabolism of alternative substrates as a compensatory response,7 and blood flow may remain unchanged.8 Others, however, have noted a hyperemic response to hypoglycemia in the perinatal brain.10–12

Thus, we undertook the current studies to test two related hypotheses. First, we hypothesized that a compensatory increase in retinal blood flow would occur in response to hypoglycemia in the perinatal period. Second, to provide data regarding the mechanism underlying a hypoglycemia-induced increase in retinal blood flow, if it occurs, we tested the hypothesis that the purine metabolite adenosine is involved in eliciting this hyperemia. Stimulus-induced changes in arteriovenous transit times and arteriolar and venular diameters, measured by image analyses of fluorescein videoangiograms from anesthetized newborn piglets, were used to calculate changes in retinal blood flow.
MATERIALS AND METHODS

Surgical Preparation

This study was approved by the Animal Studies Committee at Washington University, and it adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ten piglets, each weighing 1 to 2.5 kg and each younger than 4 days of age, were used in this study. Animals were fasted for 2 hours before surgery, but they were allowed free access to water. Animals were premedicated, tracheostomized, mechanically ventilated with a mix of room air and oxygen, and anesthetized with 1% to 1.5% isoflurane; FIO2 was typically 25% to 35%. Blood gases (model 158, Blood Gas Analyzer; Ciba Corning Medical Diagnostics, Medfield, MA), hematocrit, blood glucose concentration (Blood Glucose Analyzer; Yellow Springs Instrument, Yellow Springs, OH), and mean arterial blood pressure (internally calculated and recorded on an RS 3400 recorder; Gould, Cleveland, OH) were regularly monitored through a brachial arterial catheter. Two femoral arteries and a vein were exposed and catheterized. One artery was used to withdraw blood samples for periodic determination of blood gases, and, in the other, an arterial embolectomy catheter (4 French; Baxter Healthcare, Deerfield, IL) was inserted and advanced into the abdominal aorta; the balloon was progressively inflated as needed to maintain mean arterial blood pressure at normal levels throughout the experiment. Infusion of 5% dextrose in 0.45% NaCl (5 ml/kg per hour, intravenously [IV]) through the femoral venous catheter was used to maintain body fluid balance. End-tidal CO2 was continuously monitored (78356A Capnometer; Hewlett-Packard, Palo Alto, CA). Rectal temperature was kept at 38°C to 39°C by a thermoregulated heating pad placed under the animal. The animal’s head was stabilized within a customized aluminum cradle, the eyelids were gently retracted by sutures, the pupil was dilated (1% tropicamide), and pancuronium bromide was administered (0.5 mg/kg, intramuscularly, loading dose; 0.25 mg/kg per hour, IV, maintenance dose) to immobilize the extraocular muscles during the experimental period.

Fluorescein Videoangiography

To optimize resolution of the retinal microcirculation for fluorescein angiography, we designed a unique irrigating corneal lens device. This consisted of a 12-mm round glass coverslip (#0; VWR Scientific, West Chester, PA) at the end of an irrigating stainless steel arm held by a micromanipulator; when the coverslip was advanced close to the cornea, 0.45% saline dripping from an inverted 1-l IV bag filled the space between the cornea and the coverslip, providing a flat surface for fundus observation by epifluorescence microscopy. By passing the saline through gas permeable tubing before reaching the irrigating lens device, loss of optical clarity from corneal edema secondary to corneal hypoxia was avoided, and high-resolution angiograms could be obtained in an intact eye.

We also modified the conventional approach to retinal fluorescein angiography by injecting fluorescein (0.04% sodium fluorescein; Sigma Chemical, St. Louis, MO) directly into the external carotid, which, in pigs, leads to the ophthalmic artery.13 The common carotid artery and its internal and external branches ipsilateral to the eye studied were exposed, and the external carotid was catheterized with a 22-gauge angiocatheter for this injection. An automatic syringe pump (Harvard Apparatus, South Natick, MA) was used to standardize the injection (50 to 150 μl bolus at a rate of 25 ml/min). Because no laminar flow of fluorescein was observed, we assume the dye mixed well with the arterial blood before entering the retinal vasculature. By using close intraarterial injections of fluorescein rather than the traditional intravenous route, much lower fluorescein concentrations sufficient for angiography allow for repeated angiography (more than 30 angiograms at a rate of ≈1 every 10 seconds, if desired). Concomitantly, the progressive staining of the vascular wall common to intravenously injected fluorescein solutions of higher concentration were avoided.

The epifluorescence stereomicroscope (model BHMG; Olympus, Lake Success, NY) was mounted on a flexible boom stand for precise positioning above the preparation. Adjacent arteriolar and venular branches in the superior quadrant, 1 to 2 disc diameters from the margin of the optic disc, were chosen for measurement of fluorescein transit times and vessel diameters. Retinal angiograms were captured at 140× by a newicon camera (WV-1550; Panasonic, Secaucus, NJ) and video-recorder (SuperVHS Model HR-S8000U; Victor Company of Japan, Elmwood Park, NJ).

Arteriovenous transit times and arteriolar and venular diameters were calculated offline from the videotape record using a high-resolution monitor (PVM1343MD; Sony, Midvale, NY) and public domain image analysis software (Image, version 1.47; W. Rasband, National Institutes of Health). The difference integral method was used to calculate the mean arteriovenous transit time, as described in detail previously.14 In brief, video-recordings were digitized (Scion capture card), and gray scale values of specific, user-defined areas within the middle two thirds of each vessel were determined at 30-msec intervals; gray scale intensity before arrival of fluorescein was taken as zero. Then, the area under each intensity curve was integrated and subsequently normalized to the respective maximum values. The difference, in seconds, between the respective half-maximal points on the resultant arteriolar and venular curves was taken as the arteriovenous transit time (see Fig. 1). In the five animals comprising the hypoglyc-
FIGURE 1. Measurement of mean arteriovenous transit time from a representative videoangiogram from the piglet fundus. Arteriolar values are shown as open circles and venular values as filled squares. The upper panel shows the raw data fluorescein transits within arteriole and venule wherein the intensity (arbitrary units) before the arrival of fluorescein was taken as zero. Because of the intracarotid injection of fluorescein, there was no need to correct for tracer recirculation on the downslope of the transits as is necessary with intravenously administered fluorescein. The lower panel shows the resultant curves after integrating the area under each fluorescence intensity curve and normalizing the asymptotic plateau value for each vessel to 1.0. Mean arteriovenous transit time was calculated as the time interval (arrows) between the half-maximal points on the respective normalized intensity functions, which, in this angiogram, was 0.72 second.

In the hypoglycemia group, we found that three repeated measurements of the arteriovenous transit time at 10-minute intervals during baseline conditions yielded a coefficient of variation (standard deviation/mean × 100%) of 5.8 ± 1.1%, placing a high measure of confidence in stimulus-induced changes in transit time that are greater than 6%.

For vessel diameter measurements, the diameters of the arteriolar and venular branch pair, in which fluorescein transits and diameters were determined, varied from animal to animal because of random variations in vascular branching anatomy, animal weight, and optimization of fluorescein filling. Typically, for arterioles, secondary branches were chosen; for venules, the smaller secondary or tertiary branches that filled homogeneously with fluorescein were intentionally selected for measurement to avoid the laminar flow of fluorescein that occurs in the larger venules. Just after the peak in vessel fluorescence, vessel diameters were measured repeatedly in a single video frame (three times per frame) by an observer blinded to the identity of the experimental groups, and the mean diameter was recorded. In the hypoglycemia group of five animals, the coefficient of variation for three repeated diameter measurements of the same arteriole and venule was 1.2 ± 0.2% and 1.6 ± 0.3%, respectively, reflecting our high degree of videomicroscopic resolution. When vessel diameters were measured three times at 10-minute intervals during baseline conditions in these five animals, coefficients of variation of 3.3 ± 0.3% and 2.5 ± 0.5% were calculated for the arteriole and venule, respectively; these data indicate that stimulus-induced changes in vessel diameters greater than 3% can be accepted with a high degree of confidence.

From the arteriolar and venular diameter data and the arteriovenous transit times, retinal blood flow was calculated by the following formula:

\[ \text{Retinal blood flow} = \frac{\left( \text{arteriolar diameter} \right)^2 + \left( \text{venular diameter} \right)^2}{\text{arteriovenous transit time}} \]

Protocols

Piglets were divided randomly into two groups of five animals each. Baseline measurements of hemodynamic and physiologic variables, as well as fluorescein angiograms, were obtained at 10-minute intervals over a minimum 40-minute baseline control period in all animals. After the baseline period, hypoglycemia was induced with insulin (25 IU/kg, IV); blood glucose concentrations below 25 mg/dl (1.4 mmol/l), which occurred during the next 44 ± 7 minutes (n = 5 animals), were taken as indicative of moderate-to-severe hypoglycemia. Twenty minutes (actual time = 16 ± 3 minutes in the five experimental animals) before blood glucose levels fell below 25 mg/dl, which we estimated based on extensive experience in this hypoglycemia model, the control animal group was administered saline (10 ml), and the experimental group received the nonspecific adenosine receptor antagonist 8-phenyltheophylline (8PT; 10 mg/kg, IV). 8PT was prepared as a 1% dimethyl sulfoxide solution in 10 ml saline with a pH of 12.4 ± 0.07; this solution was administered at a rate of 1 ml/minute; blood pH was unaffected at 10 minutes after the administration of the alkalotic 8PT solution (7.33 ± 0.04 before 8PT and 7.30 ± 0.03 after 8PT). Angiographic measurements were repeated as in the control group every 10
### TABLE 1. Physiologic Variables in the Two Animal Groups

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>MABP (mm Hg)</th>
<th>Blood Glucose (mg/dl)</th>
<th>Arterial pH</th>
<th>PAO₂ (mm Hg)</th>
<th>PACO₂ (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>59 ± 2</td>
<td>64 ± 9</td>
<td>7.36 ± 0.03</td>
<td>33 ± 2</td>
<td>104 ± 4</td>
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<tr>
<td>HG</td>
<td>59 ± 3</td>
<td>20 ± 1*</td>
<td>7.28 ± 0.02</td>
<td>36 ± 2</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Baseline</td>
<td>65 ± 6</td>
<td>88 ± 5†</td>
<td>7.38 ± 0.01</td>
<td>33 ± 2</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>HG + 8PT</td>
<td>60 ± 4</td>
<td>19 ± 3*</td>
<td>7.29 ± 0.02</td>
<td>36 ± 2</td>
<td>107 ± 4*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, with n = 5 for each animal group.
HG = hypoglycemia; 8PT = 8-phenyltheophylline; MABP = mean arterial blood pressure; PAO₂ = arterial carbon dioxide tension; PACO₂ = arterial oxygen tension.
* P < 0.05 versus baseline in same animal group (paired t-test).
† P < 0.05 versus untreated group (unpaired t-tests on normalized data).

### RESULTS

Physiological variables in the two animal groups are shown in Table 1. Baseline values were not different between the two animal groups, except that blood glucose levels in the 8PT group were slightly higher than the control group. Except for a slight reduction in arterial pH in both groups, no significant changes occurred in blood pressure, blood gases, or hematocrit (data not shown) as a result of rendering the animals hypoglycemic.

Actual arteriovenous transit time data and vessel diameter data for the two animal groups is provided in Table 2, which shows both the actual values and the normalized values for these variables. In the untreated animal group (n = 5), the mean increase in retinal blood flow is shown. Differences in arteriolar diameters between the two animal groups rendered comparisons of actual flows meaningless (see Discussion). All data are shown as mean ± SEM. Differences were considered significant at P < 0.05.

### TABLE 2. Retinal Hemodynamic Data in the Two Animal Groups

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Actual Values (sec or μm)</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>HG (10 min)</td>
</tr>
<tr>
<td>Arteriovenous transit time</td>
<td>0.95 ± 0.06</td>
<td>0.69 ± 0.10*</td>
</tr>
<tr>
<td>HG</td>
<td>1.02 ± 0.19</td>
<td>0.92 ± 0.19</td>
</tr>
<tr>
<td>HG + 8PT</td>
<td>129 ± 12</td>
<td>146 ± 13*</td>
</tr>
<tr>
<td>Arteriolar diameter</td>
<td>180 ± 10†</td>
<td>194 ± 8*†</td>
</tr>
<tr>
<td>HG</td>
<td>129 ± 20</td>
<td>140 ± 19</td>
</tr>
<tr>
<td>HG + 8PT</td>
<td>153 ± 15</td>
<td>157 ± 12</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, with n = 5 for each group.
HG = hypoglycemia; 8PT = 8-phenyltheophylline.
* P < 0.05 versus baseline in same animal group (paired t-tests on actual data).
† P < 0.05 versus untreated group (unpaired t-tests on normalized data).
blood flow measured during the initial 30 minutes of hypoglycemia was 94 ± 18% (P < 0.002). In particular, retinal blood flow increased by 80 ± 17% (P = 0.03), 104 ± 24% (P = 0.02), and 100 ± 15% (P = 0.003) at 10, 20, and 30 minutes of hypoglycemia, respectively (Fig. 2). In this animal group, mean transit time decreased by 31 ± 7% (P = 0.003), and mean arteriolar and venular diameters increased by 16 ± 2% (P = 0.0007) and 11 ± 4% (P = 0.03), respectively, during the initial 30 minutes of hypoglycemia. In three of these animals, retinal blood flow measurements were continued for 60 minutes of sustained hypoglycemia, at which time flow was still significantly elevated by 72 ± 25% (P = 0.04).

Pretreatment with 8PT significantly attenuated the hypoglycemia-induced increase in retinal blood flow (Fig. 2). In these five animals, hypoglycemia only increased retinal blood flow by 24 ± 11% (P = 0.08), 16 ± 9% (P = 0.12), and 19 ± 16% (P = 0.27) at 10, 20, and 30 minutes of hypoglycemia, respectively; note that these changes were not significantly different from baseline blood flow values in this animal group. Mean arteriovenous transit time only decreased 4% ± 8% (P = 0.81 versus baseline; P = 0.02 versus untreated group [comparing normalized data]) during hypoglycemia in 8PT-treated animals, and hypoglycemia-induced increases in mean arteriolar and venular diameters were only 8 ± 2% (P = 0.01 versus baseline; P = 0.04 versus untreated group [comparing normalized data]) and 1 ± 3% (P = 0.97 versus baseline; P = 0.04 versus untreated group [comparing normalized data]), respectively. In four of these 8PT-pretreated animals, retinal blood flow was only 7 ± 9% (P = 0.99 versus baseline; P = 0.06 versus untreated group [comparing normalized data]) above baseline at 60 minutes of hypoglycemia.

**DISCUSSION**

In the current study in the intact eye of the newborn piglet, we found that hypoglycemia was associated with a significant increase in retinal blood flow. Intravenous pretreatment with the adenosine receptor antagonist 8PT significantly attenuated this response. These results indicate that a compensatory increase in retinal blood flow occurs after acute hypoglycemia and that the purine metabolite adenosine is involved in mediating this hyperemia.

Although hypoglycemia may affect retinal function adversely, there are no reports comparable to those in brain that describe hypoglycemia-induced tissue injury in retina. Similarly, it is unknown if vascular dysfunction and loss of autoregulatory capacity occurs in retina under hypoglycemic conditions, but these phenomena are well described in the cerebral circulation. In fact, before the current study, the basic response of the retinal circulation to hypoglycemia has been explored only in an adult animal model, wherein blood velocity in large retinal veins increased soon after blood glucose levels fell below 2.2 mmol/1 (a moderate level of hypoglycemia relative to our 1.1 mmol/1 [19 mg/dl] concentration). Our current results show that a significant increase in retinal blood flow also occurs in the retina during acute hypoglycemia in the perinatal period. Compensatory increases in cerebral blood flow in response to acute hypoglycemia have been described in newborn animals; indeed, recent studies from our laboratory have confirmed this finding in the newborn pig. That a retinal hyperemia results from induction of hypoglycemia is not altogether unexpected because retinal autoregulation is well described with respect to reductions in oxygen supply. Although mobilization of retinal glycogen and vitreal glucose may provide sufficient glucose for normal retinal function during the initial minutes of a reduction in blood glucose, because insulin induces hypoglycemia progressively (45 ± 7 minutes in our animals), it is possible that glycogen was depleted and vitreal stores became severely limited by this time; thus, an increase in retinal blood flow is the primary mechanism to increase retinal glucose availability under such conditions.
Adenosine and Retinal Hypoglycemic Hyperemia

Having demonstrated a compensatory hyperemic response to hypoglycemia, we also sought evidence that the purine metabolite adenosine was involved in mediating this hyperemic response. Previous studies in our laboratory have provided data indicating that adenosine is a potent dilator of the retinal vasculature in the newborn pig, consistent with observations reported for adult animals when adenosine or its agonists are administered intravitreally. Intravenous adenosine was found to be without effect on retinal blood flow, which is likely the result of an active metabolism of adenosine by endothelial cells. Based on results from an intravitreally delivered adenosine antagonist, we also concluded that that adenosine contributes to autoregulatory dilations of retinal arterioles in response to hypoxia and in response to reductions in retinal perfusion pressure. In the current study, we used an intravenous route to administer the adenosine antagonist 8PT to provide better distribution of this nonpolar antagonist throughout the retinal vasculature than would have been achieved with an intravitreal injection. In a preliminary study, intravenous administration of the nonspecific adenosine receptor antagonist aminophylline was effective in attenuating the hypoxic hyperemic response in cat retina. In piglets, the concentration of 8PT in plasma and cerebrospinal fluid was constant for 30 to 60 minutes after the administration of 8 mg/kg, IV; this coincided with the period of time during which our angiographic measurements of hypoglycemic retinal blood flow were obtained, indicating that a stable concentration of this competitive antagonist was present at retinal vascular adenosine receptors throughout the acute hypoglycemic period. Although dilation of retinal resistance vessels by adenosine is likely mediated by the A2 subtype of adenosine receptor, 8PT is a nonspecific antagonist capable of antagonizing adenosine’s action at A2 as well as A1 receptors; as discussed earlier, the A1-receptor-mediated increase in retinal metabolism that might concomitantly occur after 8PT administration would be expected to exacerbate, not diminish, the hyperemic response to hypoglycemia. In any case, the current finding that 8PT severely attenuated the increase in retinal blood flow that normally accompanies hypoglycemia indicates that adenosine is important in mediating autoregulatory adjustments of the retinal circulation to reductions in glucose supply. Other metabolic factors that may contribute with adenosine to this hyperemic response have not yet been identified.

Adenosine is metabolically well positioned to mediate a compensatory hemodynamic response to retinal hypoglycemia for the following reasons. As use of available and mobilized glucose becomes limited, adenosine triphosphate levels will fall, with the resultant production of adenosine. By increasing retinal blood flow and, hence, retinal glucose supply, adenosine serves to counteract the effects of systemic hypoglycemia. In addition, there is evidence that adenosine increases glucose availability by promoting hydrolysis of Muller cell glycogen. Other as yet undescribed actions of adenosine may contribute to the maintenance of retinal function under hypoglycemic conditions. Although there were no significant differences in arteriovenous transit times or venular diameters between the two groups during baseline conditions, arterioles in the 8PT group were larger in diameter. Together with slightly larger venular diameters in the 8PT group, calculated baseline blood flow was 73% higher (P < 0.02) in 8PT-pretreated animals (62.9 ± 3.9 arbitrary units) compared to that calculated for untreated animals (36.4 ± 5.5 arbitrary units). Because all stimulus-induced changes in retinal blood flow were normalized within each animal to its respective baseline flow, the magnitude of the baseline blood flow in each group is not as important as the relative change in blood flows that were realized in the two groups in response to hypoglycemia or hypoglycemia with 8PT pretreatment. Nevertheless, because vascular reactivity can exhibit size dependencies under certain conditions, we cannot rule out the possibility that a reduced reactivity of the larger vessels of the 8PT group, relative to the smaller vessels of the control group, contributed to the attenuated hyperemic response to hypoglycemia we observed in the 8PT-treated group.

In summary, fluorescein videangiographic analyses in intact eyes of newborn pigs indicate that acute hypoglycemia causes an autoregulatory increase in retinal blood flow, achieved by a reduction in arteriovenous transit time as well as an increase in arteriolar diameter. Because the hyperemic response was nearly abolished after adenosine receptor blockade, we conclude that increases in endogenous adenosine concentration and activation of vascular adenosine receptors, most likely of the A2 subtype, contribute to the mediation of this hyperemic response.

Key Words

adenosine, angiography, fluorescein swine, hypoglycemia, neonate, 8-phenyltheophylline, retinal blood flow

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References


