Effects of crocetin on pulmonary gas exchange in foxhounds during hypoxic exercise

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Received 17 May 1999; accepted in final form 14 February 2000

Wagner, Peter D., Connie C. W. Hsia, Ruchi Goel, James M. Fay, Harrieth E. Wagner, and Robert L. Johnson, Jr. Effects of crocetin on pulmonary gas exchange in foxhounds during hypoxic exercise. J Appl Physiol 89: 235–241, 2000.—The carotenoid compound crocetin has been hypothesized to enhance the diffusion of O2 through plasma, and observations in the rat and rabbit have revealed improvement in arterial PO2 when crocetin is given. To determine whether crocetin enhances diffusion of O2 between alveolar gas and the red blood cell in the pulmonary capillary in vivo, five foxhounds, two previously subjected to sham and three to actual lobectomy or pneumonectomy, were studied while breathing 14% O2 at rest and during moderate and heavy exercise before and within 10 min after injection of a single dose of crocetin as the trans isomer of sodium crocetinate (TSC) at 100 μg/kg iv. This dose is equivalent to that used in previous studies and would yield an initial plasma concentration of 0.7–1.0 μg/ml. Ventilation-perfusion inequality and pulmonary diffusion limitation were assessed by the multiple inert gas elimination technique in concert with conventional measurements of arterial and mixed venous O2 and CO2. TSC had no effect on ventilation, cardiac output, O2 consumption, arterial PO2/saturation, or pulmonary O2 diffusing capacity. There were minor reductions in ventilation-perfusion mismatching (logarithm of the standard deviation of perfusion fell from 0.48 to 0.43, P = 0.001) and in CO2 output and respiratory exchange ratio (P = 0.05), which may have been due to TSC or to persisting effects of the first exercise bout. Spectrophotometry revealed that TSC disappeared from plasma with a half time of ~10 min. We conclude that, in this model of emphysema (2), arterial PO2 was restored to normal after 4 wk of daily administration of crocetin at 0.5 mg ip. Similar data were obtained in normal anesthetized rabbits (10). In hemorrhaged rats, cerebral tissue PO2 measured by microelectrodes was 7.6 Torr after a single intravenous bolus of crocetin (16) compared with 3.2 Torr in untreated but hemorrhaged rats. In other hemorrhaged rats, crocetin injected into a carotid artery resulted in 100% survival, whereas control animals suffered 50% mortality (6, 8). It was proposed that the physiological basis of these remarkable effects is enhanced diffusion of O2 in plasma and in vitro evidence for such an effect was presented (6), although the physicochemical basis remains obscure (5).

Although these results in intact animals appear to show overall enhancement of O2 supply to tissues, the physiological mechanism(s) remains to be determined. The experimental approaches in these studies lack the discrimination to discern between effects of crocetin on many of the steps in the O2 transport chain from the environment to the mitochondria.

Because the proposed mechanism is enhanced diffusion of O2 through plasma, it would make sense to examine the effects of crocetin in a diffusion-limited setting. Thus, if diffusive equilibrium between alveolar gas and pulmonary end-capillary blood exists, enhancing plasma diffusion of O2 would not be expected to improve O2 transport. Because diffusion limitation is not thought to play a role in the hypoxemia of emphysema, at least in humans (1, 14, 15, 19), it is difficult to understand the basis of improved arterial PO2 in the emphysematous rat (2). Perhaps crocetin stimulated ventilation or altered ventilation-perfusion (V/A)Q relationships in some manner. Similarly, in the hemorrhaged rats (16), tissue perfusion or its distribution, rather than diffusion, may have been improved to explain the higher tissue PO2 values.

Because of these uncertainties, we designed a study to directly test the hypothesis that crocetin improves in a variety of circumstances. In the papain-treated rat model of emphysema (2), arterial PO2 was restored to normal after 4 wk of daily administration of crocetin at 0.5 mg ip. Similar data were obtained in normal anesthetized rabbits (10). In hemorrhaged rats, cerebral tissue PO2 measured by microelectrodes was 7.6 Torr after a single intravenous bolus of crocetin (16) compared with 3.2 Torr in untreated but hemorrhaged rats. In other hemorrhaged rats, crocetin injected into a carotid artery resulted in 100% survival, whereas control animals suffered 50% mortality (6, 8). It was proposed that the physiological basis of these remarkable effects is enhanced diffusion of O2 in plasma and in vitro evidence for such an effect was presented (6), although the physicochemical basis remains obscure (5).

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Because of these uncertainties, we designed a study to directly test the hypothesis that crocetin improves
arterial Po2 through enhanced diffusion. We used doses similar, per kilogram of body weight, to those reported in the studies of Gainer et al. (8, 10, 16). We chose the exercising foxhound in which to study gas exchange, because, in hypoxia, this species becomes greatly diffusion limited in its pulmonary gas exchange (11). This is seen even in the intact foxhound but is more pronounced after recovery from partial lung resection. We used the multiple inert gas elimination technique (MIGET) (4, 20) to distinguish among the various causes of hypoxemia: 1) diffusion limitation, 2) V<sub>AV</sub>/Q<sub>AV</sub> inequality, 3) shunt, and 4) hypoxemia from relative hypoventilation during exercise.

In summary, we were unable to show any effect of crocetin on arterial oxygenation, despite conditions of considerable alveolar-capillary O2 diffusion limitation.

**METHODS**

Five foxhounds were used in the study, which had been approved by the University of Texas Southwestern Medical Center Animal Use Committee. Two of the animals had previously undergone bilateral lobectomy, one had undergone right pneumonectomy, and the other two were sham-operated controls with normal lungs. All were males, and their average weight was 23.5 kg.

**Outline of the protocol.** All studies were carried out with the dogs breathing 14% O<sub>2</sub> to magnify O<sub>2</sub> diffusion limitation of pulmonary gas exchange. Each dog was studied twice, with 1 h separating the two experiments. After catheterization (see below), data were collected in duplicate 1) at rest, 2) during moderate exercise, and 3) during heavy exercise. For all dogs, moderate exercise consisted of running at 6 miles/h on a horizontal treadmill. Heavy exercise involved running at 8 miles/h at inclines of 0–15% depending on the capabilities of each dog. The target load for heavy exercise was to attain the highest power output sustainable for the 5 min required to reach a steady state and then to complete sampling procedures. The two executions of this protocol were purposely identical to each other in speed, incline, and duration. The first run was the control experiment, in which only the phosphate buffer used to dissolve crocetin was given; the second run was commenced <5 min after the intravenous injection of 100 µg/kg of crocetin given as the trans isomer of sodium crocetinate (TSC). Given a blood volume of 70–100 ml/kg, initial blood concentration would be 0.7–1.0 W/mg/ml. In three of the dogs, the disappearance of TSC from plasma was followed spectrophotometrically for 20–30 min with use of the Kelman algorithms (12, 13) over all 12 (6 arterial and 6 venous) samples before administration of TSC. The same process was used to separately calculate P<sub>50</sub> from the 12 blood samples taken during the second exercise run after crocetin administration.

The alveolar gas equation was used to compute the alveolar-arterial Po2 difference (A-A Po2) from the temperature-corrected arterial Po<sub>2</sub> and P<sub>CO2</sub> values and the measured respiratory exchange ratio.

The MIGET was utilized in the manner described previously (4, 11). Duplicate 20-ml mixed expired samples were collected at the same time as single 6-ml arterial blood samples at each of the six times of data collection (rest and moderate and heavy exercise) before and after administration of TSC. Because of time limitations, which prevented blood sampling from the Swan-Ganz catheter, measured values of ventilation and cardiac output were used to compute pulmonary arterial inert gas levels by mass balance from the expired and arterial levels. From the resulting retention and excretion ratios of the six gases (SF<sub>6</sub>, ethane, cyclopropane, enflurane, ether, and acetone), moments of the V<sub>AV</sub>/Q<sub>AV</sub> distribution were computed in the standard manner. We used the second moment about the mean on a logarithmic scale as the index of V<sub>AV</sub>/Q<sub>AV</sub> dispersion. In addition, arterial Po<sub>2</sub> was predicted from the MIGET-derived V<sub>AV</sub>/Q<sub>AV</sub> distribution and compared with the measured arterial Po<sub>2</sub>. When the former exceeded the latter, indicating causes of hypoxemia over and above 1) V<sub>AV</sub>/Q<sub>AV</sub> inequality, 2) shunt, and 3) inadequate ventilation, a whole lung diffusing capacity (D<sub>L</sub>CO) was calculated from the algorithm of Hammond and Hempleman (9). Especially in hypoxia, when the measured arterial Po<sub>2</sub> is less than that predicted via MIGET, alveolar-capillary diffusion limitation is the generally accepted explanation, although post-pulmonary shunting could in theory also account for such a
difference (18). The calculation of DL\textsubscript{O\textsubscript{2}} explicitly assumes that, regionally, diffusing capacity is distributed in proportion to local blood flow. Accordingly, this estimate of DL\textsubscript{O\textsubscript{2}} is the lowest whole lung value that will account for the difference between measured and predicted values of arterial PO\textsubscript{2}. In the context of the present study, DL\textsubscript{O\textsubscript{2}} is a lumped parameter that per se cannot ascribe specific resistance to diffusion to any component of the O\textsubscript{2} transport pathway between alveolar gas and Hb within the red blood cell. However, comparison of values before and after TSC will reveal any change in overall conductance for O\textsubscript{2} across the lung independent of V\textsubscript{A}/Q\textsubscript{A} inequality, shunt, and ventilation.

Spectrophotometric analysis of TSC in plasma. Remaining arterial blood samples used for MIGET analysis or blood-gas measurement were centrifuged at 4,200 rpm for 10 min, and the plasma was removed and protected from light. Diluted TSC and plasma before TSC injection were also obtained, and all samples were scanned in a spectrophotometer (model DU-70, Beckman). Absorbance at 540 nm (where TSC shows no activity) and absorbance at 450 nm (where TSC shows peak activity) were measured. The value at 450 nm was corrected for the (non-TSC) signal appearing at 540 nm to yield a value reflecting the contribution of TSC to the 450-nm signal. From semilogarithmic plots of absorbance as a function of time after TSC injection, plasma half time was computed in three dogs.

Statistical analysis. Repeated-measures ANOVA was used to assess the effects of TSC and exercise on the principal outcome variables pertaining to gas exchange, with \(P \leq 0.05\) set as the discriminating level of significance.

RESULTS

Global variables: ventilation, cardiac output, VO\textsubscript{2}, and VCO\textsubscript{2}. Figure 1 shows the relationships between VO\textsubscript{2} and VCO\textsubscript{2} before and after TSC at rest and during moderate and heavy exercise. VO\textsubscript{2} was not affected by TSC, with values at each metabolic rate on the identity line. However, VCO\textsubscript{2} appeared systematically reduced, albeit to a minor degree. Although this reduction failed to reach significance, the ratio of VCO\textsubscript{2} to VO\textsubscript{2} (i.e., the respiratory exchange ratio) was systematically lower after TSC by 0.1 unit (\(P = 0.05\)).

Figure 2 shows ventilation, tidal volume, cardiac output, and heart rate, each as a function of VO\textsubscript{2}, and demonstrates no effects of TSC. Neither systemic- nor pulmonary artery-cardiac output relationships were affected by TSC (not shown).

Figure 3 shows arterial PO\textsubscript{2} (measured and predicted), arterial PCO\textsubscript{2} (measured and predicted), and A-aPO\textsubscript{2} (measured and predicted) before and after TSC. These relationships are plotted against VO\textsubscript{2} and show that 1) TSC had no effect on measured or predicted
values of arterial $P_{O_2}$, 2) predicted $P_{O_2}$ was considerably higher than measured $P_{O_2}$ during moderate and heavy exercise, 3) TSC was associated with an arterial $P_{CO_2}$ that was lower by 2–3 Torr ($P < 0.01$), 4) there was no difference between measured and predicted arterial $P_{CO_2}$ before or after TSC, 5) TSC had no effect on the measured A-a$P_{O_2}$ but was associated with a slightly reduced predicted value for A-a$P_{O_2}$ of 1 Torr ($P = 0.001$), and 6) during exercise, but not at rest, the measured A-a$P_{O_2}$ greatly exceeded the predicted values (18 vs. 5 Torr, $P < 0.001$), indicating that the great majority of the A-a$P_{O_2}$ is due to diffusion limitation. Figure 3 also shows that measured arterial $O_2$ saturation was not affected by TSC but fell significantly with exercise.

Figure 4 shows a small but systematic reduction in $V_{A}/Q_{O}$ inequality, as depicted by log $SD_{Q}$, the second moment of the perfusion distribution about its mean, after treatment with TSC. This explains the small reduction in predicted A-a$P_{O_2}$ of 1 Torr shown in Fig. 3. Also shown in Fig. 4 is $D_{t-o_2}$; TSC had no significant effect on this variable.

Figure 5 shows the spectrophotometric measurements of plasma absorbance in one dog, indicating TSC disappearance from the blood with a first-order process. The half time was similar among all three dogs so examined (10.9, 11.4, and 9.2 min) and was therefore ~10 min.

$P_{50}$ was 28.0 Torr before TSC and 28.0 Torr after TSC, with no significant effect of TSC.

**DISCUSSION**

Summary of major findings. In contrast to the reports from the laboratory of Gainer et al. (2, 6, 16), we were unable to confirm enhancement of pulmonary $O_2$ diffusive transport by TSC in a highly diffusion-limited situation of hypoxic exercise. We did note some minor differences between the control and post-TSC runs, but these were related to 1) changes in $V_{CO_2}$ and $CO_2$ transport and 2) changes in $V_{A}/Q$ matching.

Lack of effect of TSC. The lack of effect of TSC in the present study could have several explanations. First, the diffusion-enhancement hypothesis, in vivo, may not be true. In vivo, convective motion of red blood cells in the pulmonary circulation could overcome potential diffusive resistance of the plasma, such that the potential benefit of TSC may not be realized. Second, even if TSC enhances plasma $O_2$ movement, if that component of $O_2$ diffusive exchange between alveolar gas and capillary blood is quantitatively minor, TSC may have no measurable effect. Thus, if crossing the alveolar-capillary membrane and/or binding to Hb in the red blood cell offer the major resistances to $O_2$ exchange, the plasma enhancement may not accelerate the overall exchange process to any measurable degree. Third, the concentration of TSC may have been insufficient to
elicits an effect. It appears that TSC disappears rapidly from the plasma (Fig. 5), but even at this rate about one-half the initial concentration should have still been present at the time of the final measurement during heavy exercise. We used doses similar, per kilogram of body weight, to those used by others (M. Singer, R. P. Stidwell, A. Nathan, and J. L. Gainer, unpublished observations). Figure 4 could be interpreted as providing tantalizing evidence in this direction, since at moderate exercise the post-TSC DLO₂ is numerically greater than the pre-TSC value. Because moderate exercise was always performed sooner after TSC administration than was heavy exercise, the TSC levels must have been higher. Figures 4 and 5 do provide a rationale for repeating these studies at higher doses and, possibly, by continuous infusion to maintain plasma levels. The fourth possible reason for lack of effect of TSC is that O₂ exchange at the lungs was not diffusion limited. This is regarded as extremely unlikely given that the measured A-aPO₂ of -18 Torr exceeded the predicted value at heavy exercise by -13 Torr (Fig. 3). The only other explanation for the difference under hypoxic conditions between predicted and measured arterial Po₂ would be a postpulmonary shunt through the bronchial or thebesian venous circulation, but a shunt of >15% of the cardiac output would be required to account for the 13-Torr difference. This is unreasonably high, since such shunts are generally only on the order of 1–2% at most.

Reconciliation with previous work. The only previous reports of crocetin affecting pulmonary gas exchange (2, 6) involve conditions of rest and normoxia, in which alveolar-capillary diffusion limitation is generally not seen, especially in health, but even in diseases (1, 14, 15, 21). In the study by Holloway and Gainer (10), arterial Po₂ rose progressively over 3 h, whereas cardiac output and iliac (not pulmonary arterial) Po₂ remained constant. Factors not considered by these authors, such as progressive changes in whole body VO₂ or pulmonary V/Q relationships, might have contributed to the changes in arterial Po₂. In the emphysema-tous rats (2), levels of ventilation and/or cardiac output or degrees of V/Q mismatch may have differed between groups to account for differences in arterial Po₂; unfortunately, arterial PCO₂ values were not given. Thus definitive explanations for the differences between previous studies and the present experiment cannot be given, since in the prior work many key variables could not be measured.

Fig. 4. TSC slightly reduced ventilation-perfusion Q mismatch (by -0.05 unit) at rest and during exercise (A). TSC did not affect whole lung O₂ diffusing capacity (DLO₂; B).

Fig. 5. Exponential disappearance of TSC from plasma in 1 dog after injection of a bolus of 100 μg/kg TSC. Data represent spectrophotometric absorbance values at 450 nm (corrected for non-TSC contributions). The 446 peak value was corrected by 540 peak (no TSC) value = sample (446) - blank (446) × blank (540)/blank (540).

Fig. 6. Arterial Po₂ during heavy exercise in hypoxemia in each dog before and after TSC. Bi-LOBX, bilateral lobectomy; R-PNX, right pneumonectomy. Surgical state had no effect on the response to TSC.
We did not perform studies during normoxic exercise in these dogs. The basis for this decision was that if TSC does not improve diffusive O₂ exchange during hypoxia, it would not be expected to do so in normoxia, where diffusion limitation is considered to be less evident. However, it remains possible that in normoxia TSC could alter pulmonary gas exchange or that the different conditions in hypoxia could affect the actions of TSC. To that extent, normoxic studies would be useful in resolving the differences between our findings and those of Gainer et al.

**Differences in VCO₂ and CO₂ transport between first and second exercise bouts.** VCO₂ and the respiratory exchange ratio were systematically lower after than before TSC, and these data were mirrored by independently measured reductions in arterial and mixed venous PCO₂. Although the effects were physiologically minor (0.1-unit reduction in respiratory exchange ratio, 3- to 4-Torr reduction in PCO₂), the question arises as to whether this result was due to TSC. It may have been due to persisting metabolic effects of the prior (control) exercise run without TSC, causing a shift away from carbohydrate toward fat as a substrate for metabolism during the second run (7). It would be necessary to repeat the entire study with two “control” runs to sort this out. Although this was seriously considered, the effort was not believed to be justified given the evident lack of effect of TSC on O₂ transport, which remains the major focus of the study. Prior work from Johnson’s laboratory in similar dogs has shown no effect of ordering on cardiopulmonary variables, so the possibility exists that these are TSC-mediated effects. The CO₂ results, however, do point out a limitation of the present study design, since TSC was always studied during the second bout of exercise. The reason for not reversing the order (of giving buffer vs. TSC) was the prolonged duration of effect reported in earlier work, approaching 200 min (10).

**Differences in VA/Q inequality between the first and second exercise bouts.** VA/Q matching improved, as shown by the dispersion of the perfusion distribution (Fig. 4). This was seen at rest and during exercise, and the overall effect was highly significant \((P < 0.001)\). The quantitative improvement was very small, however, reducing log SDQ by only 0.05 unit from 0.48 to 0.73, with the overall effect being highly significant \((P < 0.001)\).

**Figure 4** explores this possibility by presenting arterial PO₂ before and after TSC during heavy exercise in each animal studied. There was no difference in the effects of TSC on arterial PO₂ as a function of surgical state. This further suggests lack of diffusive enhancement of O₂ transport by TSC.

In summary, we have failed to corroborate the several reports of enhanced O₂ transport across the lungs by crocetin, under conditions where diffusion limitation of O₂ exchange could be well demonstrated. This could reflect 1) an insufficient concentration of crocetin at the time of measurement; 2) possible enhancement by crocetin of a part of the O₂ diffusion pathway that contributes minimally to O₂ transport resistance, even in the absence of the compound; or 3) lack of effect of TSC on the diffusion of O₂ through plasma, refuting the basic hypothesis of its mechanism of action.

We thank Richard Hogg and Deborah Tuttle for assistance with animal care and training.

This work was supported by Allos Therapeutics (Denver, CO) and National Heart, Lung, and Blood Institute Grant HL-17731.

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