Effect of Graded Hypoxia on Retention of Technetium-99m-Nitroheterocycle in Perfused Rat Heart

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The purpose of this investigation was to determine the effects of graded hypoxia on the retention of a ^{99m}Tc-labeled nitroimidazole. **Methods:** Rat hearts were perfused retrogradely with Krebs-Henseleit buffer at 37°C and paced at 5 Hz. After a 20-min stabilization period, coronary flow was maintained at 8 ml/min/g wet wt and the hearts were perfused with media equilibrated with gas mixtures containing 5% CO_2 and various levels of O_2, from 544 to 29 Torr. Technetium-99m-O(PnAO-1-(2-nitroimidazole)), BMS-181321, was infused for 20 min into a side port of the aortic cannula. Perfusion continued for an additional 40 min to allow for compound clearance. **Results:** Each decrease of perfusate PO_2 brought about an increase in the retention of BMS-181321, resulting in a good correlation between its retention and perfusate PO_2 ($r = 0.97$). Myocardial oxygen consumption was independent of oxygen delivery when the perfusate oxygen pressure was greater than 350 Torr. Below this value, oxygen consumption declined markedly as influent PO_2 was decreased. By contrast, the tissue lactate/pyruvate ratio and lactate efflux rose with each decrease of influent PO_2. A good correlation was obtained between retention of the nitroheterocycle and the cytosolic lactate/pyruvate ratio ($r = 0.98$). When glucose was omitted from the perfusate (PO_2 = 27 Torr), retention of the nitroheterocycle was increased by about 25% as compared to hearts perfused in the presence of this substrate. **Conclusion:** These results indicate that myocardial retention of BMS-181321 is coupled to the level of tissue oxygenation and that hypoxic retention may be affected by substrate input.

Key Words: oxygen; ischemia; coronary blood flow; technetium-99m nitroimidazole

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Coronary vessel disease is a leading cause of death in the United States. The noninvasive identification of cardiac tissue deprived of oxygen sufficient to maintain appropriate levels of energy (ATP) would benefit clinical diagnostic procedures and therapeutic interventions. Chapman et al. (*1*) suggested that radiolabeled nitroheterocycles might be useful for identification of hypoxic tissue using either SPECT or PET. Recently, Parliament et al. (*2*) obtained planar and SPECT images of tumors in humans after intravenous injection of ^{123}I-iodoazomycin arabinoside. Identification of ischemic canine myocardium has also been possible using fluorinated derivatives (^{18}F) of misonidazole and PET (*3*).

In an effort to create a noninvasive procedure for evaluating tissue oxygenation, a ^{99m}Tc-labeled 2-nitroimidazole (BMS-181321) has been developed which is preferentially retained in hypoxic cardiac myocytes (*4*). In the latter study, it was not demonstrated what levels of oxygen deprivation were necessary to produce increased cellular localization of this nitroheterocycle. In the present study, we have examined the oxygen dependence of BMS-181321 retention in the isolated rat heart perfused with cell-free media. The data show that each decrease of oxygen pressure in the perfusate resulted in a concomitant increase in the retention of BMS-181321. Moreover, retention in hypoxic tissue was enhanced in the absence of the anaerobic substrate, glucose.

MATERIALS AND METHODS

Heart Perfusion

Male Sprague Dawley rats (300–350 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p., Nembutal, Abbott, Chicago, IL) and heparinized (500 IU, Invenex, Chagrin Falls, OH) via the caudal vena cava. Hearts were excised rapidly and perfused retrogradely in the isolated state at 37°C with Krebs-Henseleit buffer as described previously (*5–6*). All hearts were vented through the apex using an 18-gauge needle, thereby permitting drainage of any perfusate in the left ventricle. The perfusate was supplemented with 11 m*M* glucose, 0.2 m*M* pyruvate and 12 IU/liter of insulin and was equilibrated with O_2:CO_2 (95:5, normoxia). All hearts were first perfused for 20 min with normoxic media at a perfusion pressure of 72 cm water. After this initial adjustment period, perfusate flow was maintained constant at 8 ml/min/g wet wt using a pump (Cole-Parmer #7014). At this time, some hearts were continued on the normoxic perfusate whereas others were switched to a medium equilibrated with 60%, 40%, 20%, 10% or 5% O_2 and 5% of CO_2 balanced in nitrogen or to medium nominally free of oxygen (N_2:CO_2; 95:5, hypoxia) for the remainder of the experiment. For some experiments, the gas mixture was blended to provide an influent oxygen pressure of 357 ± 7 Torr,

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TABLE 1
Effect of Influent Oxygen Pressure on Retention of BMS-181321, Oxygen Consumption and Tissue Metabolites in Perfused Rat Heart

	Influent PO_2 (Torr)							
	544 ± 20	357 ± 7	347 ± 7	242 ± 5	134 ± 5	85 ± 3	57 ± 1	29 ± 1
Retention of BMS-181321 (%)	29.4 ± 2.3	35.5 ± 2.3	42.8 ± 2.3	44.8 ± 1.4	48.5 ± 4.1	52.8 ± 0.5	57.0 ± 3.5	59.0 ± 1.1
Oxygen consumption (μmole/min/g wet wt)	3.53 ± 0.16	3.65 ± 0.07	3.66 ± 0.17	3.11 ± 0.19	1.53 ± 0.04	1.00 ± 0.07	0.63 ± 0.01	0.30 ± 0.02
Lactate/Pyruvate	4.58 ± 0.86	7.67 ± 1.24	5.38 ± 1.92	8.45 ± 1.48	14.01 ± 1.76	15.88 ± 2.90	20.50 ± 2.92	18.05 ± 3.80
Lactate efflux (μmole/min/g wet wt)	0.69 ± 0.14	1.53 ± 0.24	1.45 ± 0.27	2.05 ± 0.45	3.08 ± 0.70	4.56 ± 0.56	4.66 ± 0.41	4.97 ± 1.08

Values represent means ± s.e.m. for n = 4 experiments except at an influent PO_2 value of 544 where n = 8. Influent PO_2 was measured as the perfusate entered the windkessel vessel.

pH 7.8. Although all hearts were paced continuously at 5 Hz, contractions were visibly inhibited by hypoxic perfusion. After the first 30 min of perfusion at constant flow, hearts were infused (60 μliter/min) with the ^{99m}Tc-nitroimidazole (BMS-181321, about 1.5 Ci/mmole, 100 μCi/ml) for 20 min and then perfused with medium free of radioactivity (cold) for another 40 min.

For determination of oxygen consumption, a cannula was placed in the right ventricle via the pulmonary artery. A pump (Cole-Parmer #7013) removed a small fraction (1 ml/min) of the coronary influent and effluent, and their oxygen pressures were monitored continuously by two in-line Clark-type oxygen electrodes coupled to a dual-channel oximeter. Coronary flow was measured by collecting the effluent from the right and left pulmonary arteries in a 10-ml graduated cylinder. Oxygen consumption was calculated from the product of the influent-effluent oxygen pressure difference and the coronary flow. Lactate in the coronary effluent and tissue lactate and pyruvate levels were measured as described previously (*5–6*).

Radioactivity in the perfused heart was detected by a collimated NaI crystal positioned 3–4 cm from the right ventricle and perpendicular to the vertical axis of the heart. The photocurrent was sent to a single-channel analyzer in multiscalar mode with a dwell time of 5 sec/channel. The latter instrument digitized the signal and provided real time monitoring of the experiment as previously described (*7*). The radioactivity remaining in the heart after 40 min of perfusion with tracer-free media (washout) expressed as a percent of the peak level of radioactivity at the end of the infusion period was used as a measure of retention.

Preparation of BMS-181321

Synthesis of BMS-181321 (oxo [[3,3,9,9-tetramethyl-1-(2-nitro-1H-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioximato]-(3-)-N, N′, N″, N‴]technetium) and its structure have been described previously (*4,8*). In brief, BMS-181321 was prepared by dissolving 2.0 mg of PnAO-1-(2-nitroimidazole) ligand in 1.5 ml of saline, 0.5 ml of 0.1N $NaHCO_3$ and 0.5 ml of generator eluant (up to 100 mCi $^{99m}TcO_4$-). The reaction was initiated by addition of 50 μl of a deoxygenated saturated stannous tartrate solution. The reaction was completed within 10 min at room temperature. Measurement of radiochemical purity using previously described methods (*4*) was always greater than 90%.

RESULTS

Under baseline conditions in which oxygen pressure in the coronary influent was high (544 Torr), retention of BMS-181321 was only 29.4% ± 2.3% (Table 1). By decreasing the perfusate PO_2 below this value, a marked enhancement of retention resulted (Fig. 1) for each change in oxygen delivery. Even small changes in perfusate oxygen pressure, for example from 357 to 347 Torr, were associated with altered localization of the nitroheterocycle. It should be noted, however, that in the former case, the pH of the media was slightly alkaline, pH = 7.8. At the lowest level of perfusate PO_2 (29 Torr), the amount of BMS-181321 remaining in the hearts after 40 min of clearance with cold perfusate was 59% ± 1.08% (Table 1), nearly a two-fold difference between the two extreme conditions of oxygenation. A good inverse correlation between

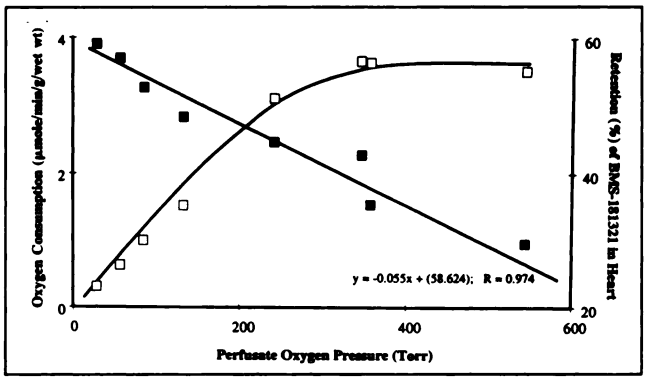

FIGURE 1. The effect of perfusate oxygen pressure on myocardial oxygen consumption and on BMS-181321 retention in perfused rat heart. The open squares represent mean values (minimum n = 4) of oxygen consumption. The hand drawn line through these values was computer assisted using commercially available software (Micrografx, Richardson, TX). The closed squares represent mean values of retention as described in Methods. The regression equation in the lower right corner and regression line relate the correlation between perfusate oxygen pressure and retention of the nitroheterocycle. Standard errors of the mean are provided in Table 1.

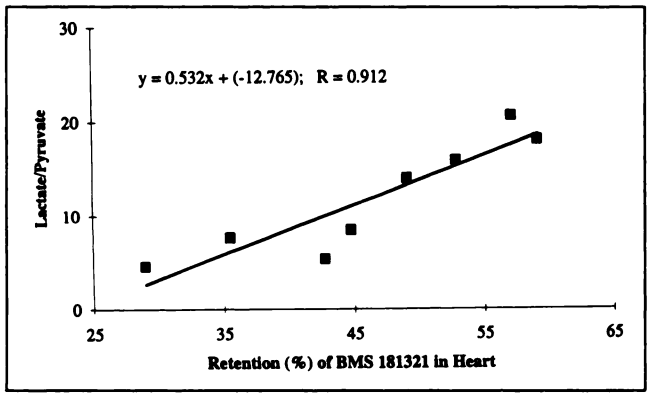

FIGURE 2. The relationship between the retention of BMS-181321 and the redox state of the cytosolic pyridine nucleotides in the perfused rat heart. The redox state of the cytosolic pyridine nucleotides is expressed as the tissue lactate/pyruvate ratio measured in neutralized extracts of freeze-clamped hearts. Hearts were freeze-clamped in aluminum tongs cooled in liquid nitrogen at the end of the perfusion period described in Methods. The regression equation is provided in the upper left corner. Standard errors of the mean (n = 4) are provided in Table 1.

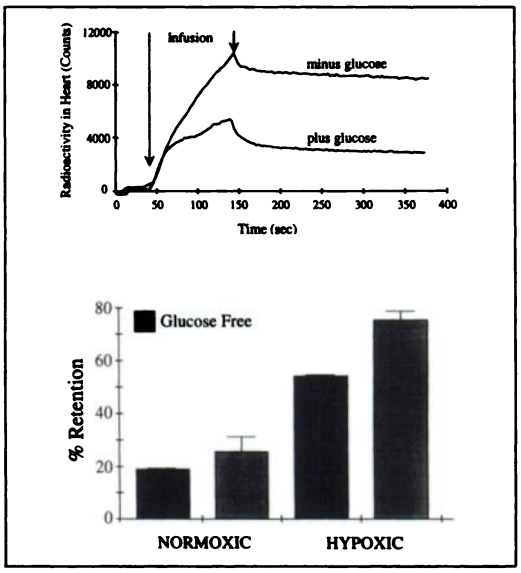

FIGURE 3. The effect of perfusate glucose on the retention of BMS-181321 in the perfused rat heart. Hearts were perfused either in the presence or absence of glucose (n = 3 for each group). Pyruvate (0.2 m*M*) was supplied in all cases. In the upper panel, a typical example of the differences in uptake and retention of hearts perfused with media nominally free of oxygen (27 Torr) and in the presence and absence of glucose. In the lower panel, mean ± s.e.m. values of retention for both normoxic and hypoxic hearts are provided.

PO_2 and retention of BMS-181321 was obtained; r = −0.97, y = −0.055X + (58.62).

When perfusate oxygen pressure was decreased from 544 Torr to either 357 or 347 Torr, oxygen consumption remained unchanged, 3.53 ± 0.16 versus 3.65 ± 0.07 and 3.66 ± 0.17 μmole/min/g wet wt, respectively (Fig. 1, Table 1). When perfusate PO_2 was decreased further, i.e., below 347 Torr, oxygen consumption was adversely affected. Below this oxygen pressure value, each lowering of perfusate PO_2 resulted in a proportional decrease in oxygen consumption.

Even though oxygen consumption was not altered by the change in oxygen supply at higher levels of perfusate PO_2, the metabolic state of the tissue changed. This was apparent by the increase in the amount of lactate efflux and by the rise in the lactate/pyruvate ratio (Table 1). When influent PO_2 was decreased from 544 to 357 Torr, lactate efflux rose from 0.69 ± 0.14 to 1.53 ± 0.24 μmole/min/g wet wt and the lactate/pyruvate ratio increased from 4.58 ± 0.86 to 7.67 ± 1.24. Additional increments in these two parameters were obtained with further lowering of perfusate oxygen pressure. At a perfusate PO_2 of 29 Torr, lactate efflux had risen to 4.97 ± 1.08 μmole/min/g wet wt and the lactate/pyruvate ratio was 18.05 ± 3.8. Moreover, Figure 2 shows that a good positive correlation (r = 0.91) was obtained between the redox state of the cytosol, expressed as the lactate/pyruvate ratio, and the retention of BMS-181321 in the heart.

The metabolic state of tissue is dependent, in part, on the level of substrate input. It was of interest, therefore, to examine the effect of perfusing hearts with media deficient in both oxygen and anaerobic fuel on the uptake and retention of BMS-181321. In the absence of glucose in the perfusate, there was more BMS-181321 taken up during the infusion period and about 25% more of the labeled compound retained after 40 min of cold washout (Fig. 3). Since there was no difference in the amount of ^{99m}Tc-DTPA retained between the two groups of hearts (data not shown), the increased retention of BMS-181321 in glucose-deficient hearts was not due to an expansion of the interstitial space. Omitting glucose from media containing normoxic levels of oxygen (>500 Torr) did not markedly affect the level of retention of the nitroheterocycle.

DISCUSSION

The major findings of the present investigation using the isolated perfused rat heart were twofold. First, the technetium-labeled nitroheterocycle, BMS-181321, was retained as the oxygen pressure in the coronary influent was decreased, resulting in an inverse linear relationship. Each change of influent PO_2 resulted in enhancement of nitroheterocycle retention. This increase in retention was obtained even though at higher oxygen pressures, i.e., above 347 Torr, myocardial oxygen consumption was unaffected by the lowering of influent PO_2. Second, at low levels of influent PO_2, the amount of retention of BMS-181321 was increased by eliminating glucose from the perfusion media.

The perfusate oxygen pressures required to maintain myocardial ATP synthesis, during baseline conditions, were markedly above what is considered to be physiological, a possible disadvantage of using an isolated heart perfused with cell-free media. There were, however, several advantages of conducting these studies using this model. For example, by perfusing the heart in the absence

of blood cells, the interaction of the radiopharmaceutical compound with serum proteins, erythrocytes or other blood cells via nonspecific effects is avoided. Consequently, uptake and retention properties of BMS-181321 were determined in an intact model containing only cells intrinsic to the heart. Previously, it was observed that other technetium ligands, more lipophilic than BMS-181321, associate avidly with blood components and that myocardial extraction of these radiopharmaceuticals was markedly affected by these interactions (*7*). Equally important, however, coronary flow and oxygen flux to the cardiac myocytes were precisely controlled in the present experiments.

The sensitivity of nitroheterocycles for intracellular oxygen and their potential for demarcation of hypoxic tissue has been the subject of recent investigation by a number of research groups (*1–3*). Nitroheterocycles, originally designed for treatment of certain bacterial infections or radiosensitizers in cancer therapy are thought to undergo enzyme-mediated intracellular reduction (*9*). These electron affinic compounds are reduced to a radical anion in both normoxic and hypoxic environments. This nitro radical species can react with oxygen to produce the superoxide anion and to regenerate the initial form of the nitroheterocycle. It is at this step that the affinity of 2-nitroimidazoles such as BMS-181321 for molecular oxygen affects the level of their retention in tissue. In hypoxic tissue, the nitro radical is thought to be reduced further (a possible total of 4–6 electrons) to yield hydroxylamine and amine derivatives (*10*) which may covalently bind to intracellular macromolecules (*11–13*). Advantage of these reactions has been made in order to identify hypoxic cells in tumors and in heart.

Data obtained from this study suggest that even small decreases in the level of oxygen flux to the cardiac myocytes can result in enhanced retention of BMS-181321. Although intracellular oxygen pressures were not determined, it can be assumed that a large oxygen pressure gradient existed between the capillaries and the cardiac myocytes (*14*). When influent PO_2 was decreased from 544 to 347 Torr, myocardial oxygen consumption was unaffected. This finding suggests that oxygen flux to the cells was sufficient to meet the demand for oxygen established by the experimental paradigm. Since the heart preparation was a nonejecting one, nominally free of preload and afterload, this finding was not unlikely. It should be noted, however, that the metabolic state of the cells was shifted to a more reduced state, i.e., an increase in the lactate/pyruvate ratio, in order to match ATP synthesis with ATP demand. These data suggest, therefore, that BMS-181321 is sensitive to oxygen in a range of values that influence the metabolic state of the tissue prior to a significant loss of metabolic function.

It was not possible to determine the precise intracellular level of oxygen necessary for half-maximal retention of BMS-181321 in the present investigation. Workers in the field have attempted to measure the oxygen sensitivity of other 2-nitroimidazoles. For example, Van Os-Corby et al. (*15*) reported that the oxygen pressures for half-maximal binding of [^{14}C]misonidazole (50 μM) to cubes (1–2 mm wide) of EMT-6 mouse fibrosarcoma and of heart were estimated to be 2.28 and 1.9 Torr, respectively. These measurements were made by placing the tissue cubes in petri dishes containing media. The dishes were housed within a leak-proof aluminum apparatus which was positioned on a shaker table within an environmental chamber thermostated at 37°C. Importantly, oxygen determinations were obtained from the gas phase. Rasey et al. (*16*) have used similar methods for obtaining data on the oxygen sensitivity of misonidazole and its fluorinated derivative. As shown in the latter study, these types of measurements are useful for comparison of the relative oxygen sensitivities of different compounds under the same set of experimental conditions. On the other hand, these types of oxygen measurements are beset with technical difficulties (*14,17–19*). Several other factors relevant to these measurements need to be considered. First, oxygen solubility and oxygen diffusivity are low in physiological media (*20*) thus establishing an oxygen gradient between the gas and liquid interface. Second, an oxygen diffusion gradient also exists between the extracellular media and the intracellular site of oxygen utilization. The magnitude of this gradient is determined by the distance oxygen must travel to reach mitochondria and other oxygen reaction sites, and by the metabolic state of the cells (*14*). Third, surrounding each cell is a stagnant layer of oxygen that is markedly affected in cell suspensions by the degree of stirring (*14,17*).

In a suspension of well-stirred quiescent adult rat cardiac myocytes, the oxygen gradient between the extracellular space and the mitochondria was found to be about 0.8 Torr during basal conditions (*14*). To this value, the stagnant layer surrounding each cell contributed about 0.15 Torr (*21*). When the level of metabolic activity rose, the oxygen gradient increased severalfold. In the absence of physical barriers to oxygen diffusion, it is therefore likely that the true value for half-maximal binding of 2-nitroimidazoles to cells is much smaller than that observed by Van Os-Corby et al. (*15*).

Comparison of the oxygen sensitivity of BMS-181321 and that of misonidazole ([^{3}H]F-misonidazole) using the preparation of the present study was attempted without success. In preliminary studies, it was not possible to obtain hypoxic differentiation in this model using [^{3}H]F-misonidazole. On the other hand, this result suggests that the inverse correlation of BMS-18321 retention, with perfusate oxygen pressure, was not due simply to an expansion of the interstital space in the oxygen deprived hearts. The more hydrophilic character of [^{3}H]F-misonidazole as compared to BMS-181321 (*4*) likely prevented marked intracellular uptake of the former under conditions of low perfusate viscosity. Shelton et al. (*22*) showed that [^{3}H]F-misonidazole was preferentially retained in an isolated rabbit heart perfused with cell-free media containing dextran. We can only speculate that the latter increased the viscosity of the perfusate, thereby affecting flow velocity and

aiding uptake of misonidazole. Nonetheless, using suspensions of cardiac myocytes isolated from adult rat heart, it was shown previously that BMS-181321 and [^{3}H]F-misonidazole display similar patterns of uptake during conditions of oxygen deprivation (*4,23*). Similar redox potentials have also been obtained for both misonidazole and BMS-181321 (*8*). The results above suggest that both misonidazole and BMS-181321 share similar sensitivity to oxygen.

It was an interesting finding that retention of BMS-181321 was markedly enhanced in hypoxic hearts when glucose was omitted from the perfusate. Changes in substrate delivery have been shown to alter the metabolic state of the perfused heart (*6,24*). Moreover, it is well known that the production of lactate serves to maintain the redox state of the cytosolic pyridine nucleotides (NAD^+/ NADH) in the oxidized form. By omitting glucose and providing pyruvate in the perfusion media, it is likely that the metabolic state of the hearts was altered as compared to those receiving glucose. We have shown previously that changes in the metabolic and energy state of the tissue were not sufficient to affect retention of BMS-181321 when oxygen availability was high (*4*). The results of the present study suggest, however, that the metabolic state of the tissue may influence nitroimidazole retention when the oxygen supply is limited.

CONCLUSION

The results of the present study have shown that even small decrements in oxygen delivery to cardiac myocytes enhance the retention of BMS-181321 and that this retention can be markedly enhanced in the absence of anerobic substrate. Limitation of oxygen availability, even in small amounts, results in increased levels of reduction of the cytochromes of the respiratory chain and thus increases the level of reduc- ing equivalents (*14,18*). We have previously shown that BMS-181321 is retained preferentially by mitochondria isolated from rat heart following incubation under hypoxic conditions (*4*). Substrate oxidations occur primarily by the reactions of mitochondrial oxidative phosphorylation. Electron transfer from substrate to nitroheterocycle may be catalyzed by any number of intracellular reductases. It is therefore interesting to speculate that in cardiac myocytes the enzymes of oxidative phosphorylation may play a role in the intracellular trapping of 2-nitroimidazoles.

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