Effect of Hypoxia on the Accumulation of Technetium-99m-Glucarate and Technetium-99m-Gluconate by Chinese Hamster Ovary Cells In Vitro

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The accumulation of ^{99m}Tc-glucarate, an agent recently reported to localize in acutely infarcted myocardium, zones of acute cerebral injury and tumors, has been compared with ^{99m}Tc-gluconate in an in vitro system of cultured Chinese hamster ovary fibroblasts. The effects on accumulation of hypoxia and competition with fructose have been studied. Both labeled glucose analogs showed a two- to threefold enhanced accumulation in hypoxic cells relative to aerobic cells. No such enhanced accumulation under hypoxia was observed for the nonsugar tracers pertechnetate and ^{99m}Tc-DTPA. The presence of 20 m*M* fructose reduced the accumulation of ^{99m}Tc-glucarate by 30% ($p = 0.067$) and ^{99m}Tc-gluconate by 40% ($p < 0.05$) in hypoxic cells, but had no significant effect in aerobic cells. These results suggest that both compounds at least partially share a common mechanism of uptake and/or accumulation with fructose.

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It has recently been shown that ^{99m}Tc-glucarate accumulates in acutely infarcted myocardium (*1*), zones of acute cerebral injury (*2*) and experimental tumors (*3*), leading to the suggestion that this complex may be useful for imaging these lesions. The mechanism of localization of this 6-carbon dicarboxylate sugar (Fig. 1) is unknown. Although glucarate transport into renal cells can be inhibited in vitro by fructose, the discordance between the biodistribution of ^{99m}Tc-glucarate and ^{18}F-2-fluorodeoxyglucose (FDG) in experimental cerebral injury suggests that ^{99m}Tc-glucarate is not acting completely as a glucose analog (*2*). However, the commonly used radiopharmaceuticals, ^{99m}Tc-gluconate and ^{99m}Tc-glucoheptonate (6- and 7-carbon monocarboxylate sugars, respectively; Fig. 1), have also been shown to be taken up in damaged myocardium (*4*), brain (*5*) and certain tumors (*6,7*), and the cellular accumulation of ^{99m}Tc-glucoheptonate can also be inhibited in vitro by fructose (*8*). It is known that the rate of glycolysis can be increased under conditions of anaerobic metabolism. To the degree that acutely ischemic myocardium or brain contains regions of low oxygen tension, such altered uptake and accumulation of glucose and its analogs might occur.

A cell culture system has been used in order to measure specific, metabolically mediated intracellular accumulation in the absence of nonspecific, extracellular binding. A comparison has been made of the effect of low oxygen tension and fructose on the accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-gluconate with single-cell suspension cultures of a Chinese hamster ovary cell line in vitro.

METHODS

Labeled Compounds

Technetium-99m-glucarate, ^{99m}Tc-gluconate and ^{99m}Tc-DTPA were prepared from in-house kits which contained 50 μmol ligand, 3 μmol gentisic acid (antioxidant) and 2.5 μmol stannous chloride. Each kit was reconstituted with 1500 MBq [^{99m}Tc] pertechnetate which had been eluted from a generator with no more than 24 hr of in-growth. The total volume in each reconstituted kit was 1.5 ml. Preparation, stability and quality control of ^{99m}Tc-glucarate have been reported previously (*9*). Radiochemical purity of each preparation was >95%.

Cells

Chinese hamster ovary (CHO) fibroblasts, sub-line AA8-4, were grown in suspension culture in spinner flasks in growth medium consisting of complete alpha minimum-essential medium (alpha-MEM) plus 10% fetal calf serum (FCS). They had a doubling time of 11-13 hr and were assayed for their viability by a colony-formation assay as monolayer cultures (*10,11*).

In order to measure the uptake and accumulation of radiotracers, exponentially growing cells were taken directly from suspension culture at 2–4 × 10^5 cells/ml. Glass vials containing 10 ml cell suspension were placed in a water bath at 37°C. The vials were capped with a stopper which had an inlet tube through which a water-saturated atmosphere of 5% CO_2 plus 95% air or

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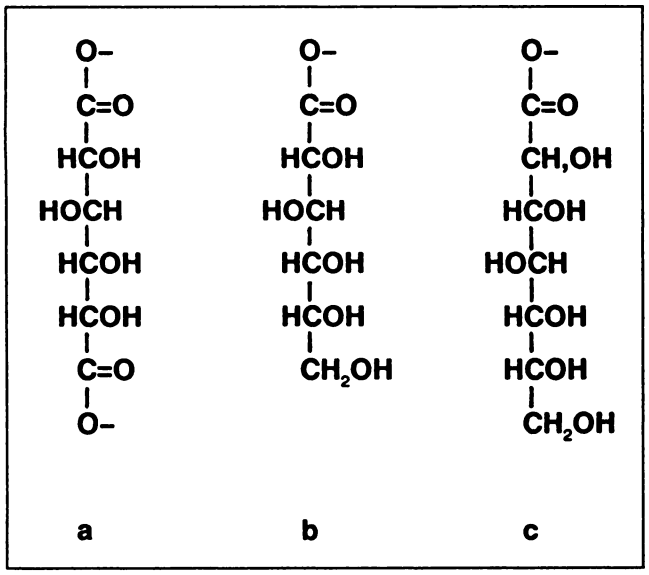

FIGURE 1. Chemical structures of: (a) glucarate, (b) gluconate and (c) glucoheptonate.

95% nitrogen (<10 PPM O_2) could be continuously flowed. An outlet port allowed the gas to escape and was used for sampling. The oxygen levels present in such suspension cultures have been documented previously and the nitrogen atmosphere has been shown to reduce the concentration of dissolved oxygen to <10 PPM within 45 min (*10,11*).

Uptake and Accumulation

An aliquot of 0.3 ml ^{99m}Tc-glucarate or ^{99m}Tc-gluconate (300 MBq, 10 μmol) was added to the 10-ml cell suspension and duplicate aliquots of 100 μl each were removed after 1, 30, 60, 120 and 180 min of incubation. The aliquots were layered over 1 ml oil (dibutyl phthalate:corn oil, 4:1) in a 1.5-ml microcentrifuge tube which was then centrifuged at 10,000 *g* for 5 min at 4°C (*12–14*). The total radioactivity in each tube was measured in a dose calibrator, then the liquid in the tube was aspirated with care to avoid any of the aqueous phase reaching the bottom of the tube. The tip of the tube, containing the cell pellet and a small amount of residual oil, was clipped off, placed in a counting tube and assayed in a gamma well counter with appropriate dilutions of a ^{99m}Tc standard. It has previously been shown in this laboratory that the error due to residual aqueous phase trapped in the oil and pellet is negligible (*14*). From the measurements of radioactivity in the cell pellet and supernatant combined with knowledge of the cell number and volume, the ratio of concentration of radioactivity per 0.1 ml of packed cells to that in 0.1 ml of the supernatant could be calculated, CPM_{in}/CPM_{out}. As a check on the specificity of drug accumulation under air versus hypoxia, control experiments were performed in a similar manner with [^{99m}Tc]pertechnetate and ^{99m}Tc-DTPA.

To ascertain whether the experimental conditions resulted in a change in cell volume, aliquots of cell suspension were analyzed with a modified Coulter electronic particle counter with a pulse height analysis attachment that allowed the frequency distribution of cell volume to be displayed and a median value calculated. There was no change in median cell volume (± 5%) as a function of aerobic or hypoxic exposure for up to 300 min regardless of cell concentration or the presence or absence of 1 m*M* glucarate.

Competition with Fructose

Since previous reports had suggested that fructose could compete with the uptake and accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-glucoheptonate, the experiments with ^{99m}Tc-glucarate and ^{99m}Tc-gluconate were repeated exactly as before, except that exponentially growing cells were spun and resuspended at 1 × 10^6 cells/ml in fresh alpha-MEM containing 10% FCS, to which 20 m*M* of fructose was added. The tracer was then added and the uptake and accumulation experiment was performed as described above.

RESULTS

The effect of the presence or absence of oxygen on the cellular accumulation of tracer as a function of time is shown in Figure 2. For both ^{99m}Tc-glucarate and ^{99m}Tc-gluconate under aerobic conditions there was a gradual increase in the ratio CPM_{in}/CPM_{out} which appeared linear over the 0–180 min incubation time. This accumulation was slightly higher for ^{99m}Tc-gluconate than for ^{99m}Tc-glucarate. Under hypoxic incubation conditions, the accumulation of radioactivity within the cell was 2–3 times higher than aerobic conditions for incubation times of 60–180 min, although there was no alteration in initial uptake as measured 1 min after addition of radioactivity. In contrast, [^{99m}Tc]pertechnetate, though showing a slow ac-

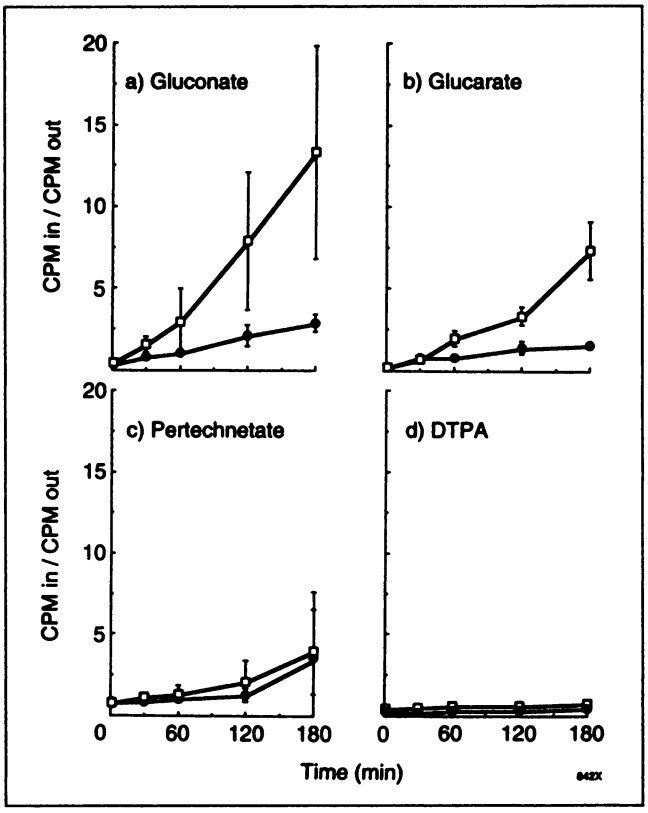

FIGURE 2. Effect of aerobic or hypoxic conditions on cellular accumulation of tracer as a function of time. (a) Gluconate, (b) glucarate, (c) pertechnetate and (d) DTPA. Aerobic, ●; hypoxic, □. Each point is mean plus or minus one standard deviation for four determinations.

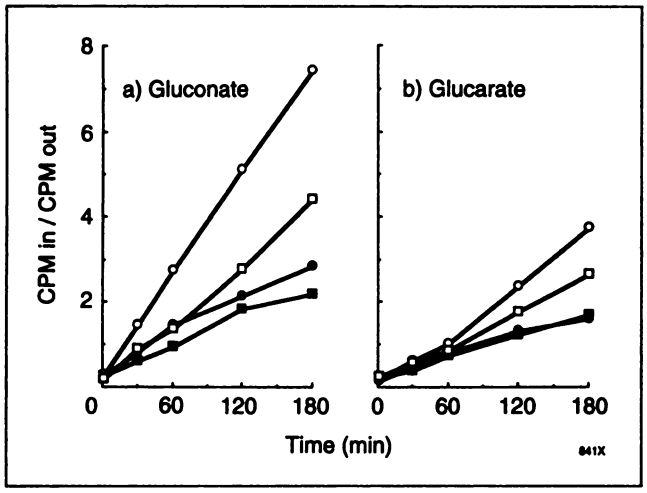

FIGURE 3. Effect of 20 m*M* fructose on accumulation of tracer by aerobic or hypoxic cells. (a) Gluconate and (b) glucarate. Aerobic control, ●; aerobic + fructose, ■; hypoxic control, ○; hypoxic + fructose, □. Each point is mean of four determinations. Standard deviation is not shown; average standard deviation is 25% of the mean.

cumulation under aerobic incubation conditions, did not show a significant increase under hypoxic conditions. ^{99m}Tc-DTPA showed much lower accumulation with time and exhibited no hypoxic versus aerobic differential. Thus only ^{99m}Tc-glucarate and ^{99m}Tc-gluconate demonstrated enhanced accumulation under hypoxia.

At the termination of two of the aerobic-hypoxic accumulation studies, the CHO cells were washed, diluted, and assayed for their colony-forming ability in vitro. In no case was there any evidence of toxicity to cells incubated with ^{99m}Tc-glucarate or ^{99m}Tc-gluconate under air or hypoxia compared to control cells with plating efficiencies near one.

The effect of the presence of 20 m*M* fructose on accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-gluconate by aerobic and hypoxic cells is shown in Figure 3. Fructose diminished ^{99m}Tc-gluconate accumulation in aerobic cells by about 20% but had no effect on ^{99m}Tc-glucarate in aerobic cells. In hypoxic cells, fructose seemed to have a greater effect, decreasing accumulation of ^{99m}Tc-gluconate by 40% (Student's t-test, $p < 0.05$) and ^{99m}Tc-glucarate by 30% ($p = 0.067$) after 180 min of incubation. Initial uptake at 1 min after addition of radioactivity was not altered by the presence of fructose.

Comparison of Figures 2 and 3, through showing qualitatively similar results for ^{99m}Tc-glucarate and ^{99m}Tc-gluconate in the absence of fructose, shows quantitative differences. Enhanced hypoxic accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-gluconate is seen in Figure 2 compared to Figure 3, though the aerobic accumulation is similar. Thus, the hypoxic versus aerobic differential was four- to fivefold in Figure 2, but only two- to threefold in Figure 3. The major difference between these two experiments is the factor of 3–5 in cell number and the fact that in Figure 3 the cells were resuspended in fresh medium versus being used directly from suspension culture in conditioned medium in Figure 2. A higher cell concentration was used for the experiments reported in Figure 3 in order to improve counting statistics and reduce the variability observed in Figure 2. Fresh medium was used in order to eliminate the variability in nutrient content and metabolic byproducts in conditioned medium. To find an explanation for the quantitative differences between Figures 2 and 3, the effects of cell number and conditioned versus fresh medium were assessed separately. Direct comparison experiments performed with conditioned or fresh medium at two cell densities demonstrated that the quantitative differences between Figures 2 and 3 are due to conditioned versus fresh medium, not cell density (data not shown). This suggests that fresh medium contains a higher level of some nutrient which competes with ^{99m}Tc-glucarate and ^{99m}Tc-gluconate and is depleted in conditioned medium. A possible candidate is glucose itself (*15*). Alternatively, conditioned medium may contain a metabolic product which enhances accumulation of the tracers.

DISCUSSION

Although there are several agents available for imaging myocardial or cerebral perfusion (e.g., ^{99m}Tc-sestamibi, ^{99m}Tc-teboroxime and ^{99m}Tc-HMPAO), there is great interest in finding a single-photon-emitting agent whose localization reflects glucose metabolism, thus allowing detection of hypoxic but viable tissue (i.e., tissue in which metabolism has become uncoupled from perfusion). Preliminary data suggested that ^{99m}Tc-glucarate might be such an agent, but further work has cast doubt upon this contention (*1,2*). Using experimental myocardial infarction in dogs, Orlandi et al. found no preferential localization of ^{99m}Tc-glucarate in hypoxic but viable myocardium. However, the agent showed high affinity for necrotic tissue, possibly due to binding to mitochondrial cytochrome oxidase (*1*). Using a middle cerebral artery occlusion/reperfusion model in rats, Yaoita et al. found intense accumulation of ^{99m}Tc-glucarate in infarcted regions but variable accumulation in viable tissue (*2*). The discordance between distribution of ^{99m}Tc-glucarate and ^{18}F-FDG was interpreted as evidence that ^{99m}Tc-glucarate does not behave as a glucose analog. Moreover, the similarity in biodistribution of ^{99m}Tc-glucarate and ^{111}In-DTPA suggested that accumulation of ^{99m}Tc-glucarate was mainly due to increased permeability of the blood-brain barrier in injured regions (*2*).

One of the present authors (*9*) and others (*1*) have noted similarities between the behavior of ^{99m}Tc-glucarate and the widely used radiopharmaceuticals, ^{99m}Tc-gluconate and ^{99m}Tc-glucoheptonate, both of which have also been shown to accumulate in damaged myocardium (*4*) and brain (*5*) and in certain tumors (*6,7*). Therefore, uptake and accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-gluconate was compared in an in vitro system of cultured cells as a

function of hypoxia and the presence or absence of excess fructose.

In control experiments, [^{99m}Tc]pertechnetate showed slow aerobic accumulation, ^{99m}Tc-DTPA showed little accumulation, and neither exhibited increased accumulation in hypoxic cells. In contrast, both ^{99m}Tc-glucarate and ^{99m}Tc-gluconate showed gradual accumulation in aerobic cells which was enhanced in hypoxic cells, with the relative enhancement (two- to threefold) similar for the two agents. These hypoxic cells were still viable; their colony-forming ability was not impaired by 3 hr of hypoxia. Their ability to exclude ^{99m}Tc-DTPA (i.e., $CPM_{in}/CPM_{out} < 1$) also shows that the cell membrane was intact, whereas Orlandi et al. had postulated that disruption of the cell membrane was required for accumulation of ^{99m}Tc-glucarate (*1*). Moreover, the difference between the enhanced accumulation of ^{99m}Tc-glucarate and ^{99m}Tc-gluconate in hypoxic cells and the lack of enhanced accumulation of ^{99m}Tc-DTPA suggests that a specific mechanism may be involved.

It has previously been shown that 20 m*M* fructose inhibits influx of ^{99m}Tc-glucarate by 61% (*2*) and accumulation of ^{99m}Tc-glucoheptonate by 88% (*8*) in renal cell line LLC-PK$_1$ under aerobic conditions. Therefore, the effect of fructose was also studied in the present system. Although little effect of fructose on accumulation of either tracer by aerobic cells was detected, under hypoxia fructose inhibited accumulation of ^{99m}Tc-glucarate by 30% ($p = 0.067$) and ^{99m}Tc-gluconate by 40% ($p < 0.05$). The lack of a significant effect under aerobic conditions may be due to the lower effective specific activity in the present studies. Yaoita et al. (*2*) used 20 m*M* fructose and 0.1 m*M* glucarate (apparent fructose molar excess 200:1), whereas in the present studies the concentration of glucarate was 1 m*M* (excess only 20:1). There could also be differences in the properties of the cell lines used, as reported for ^{99m}Tc-glucoheptonate (*8*). Moreover, previous studies reported initial entry rates, determined at 5 min, whereas the present studies report CPM_{in}/CPM_{out} ratios over periods of up to 3 hr. However, the observation of a fructose effect in hypoxic cells supports the hypothesis of a specific uptake mechanism. In this regard, evidence exists in the literature for a specific fructose transport mechanism separate from glucose, but in at least some cells fructose can enter by glucose transport as well (*16*). Thus, the exact role of specific transport systems for ^{99m}Tc-glucarate and ^{99m}Tc-gluconate remains to be established.

The present studies show many similarities between ^{99m}Tc-glucarate and ^{99m}Tc-gluconate and suggest that the two agents share a common mechanism(s) of accumulation. The differences between these agents and ^{99m}Tc-DTPA suggest that one specific mechanism involves fructose transport. The system described herein is amenable to study other factors, such as the dependence of cellular uptake and accumulation on oxygen tension, fructose-to-chelate ratio and cell type, which are important in assessing the potential clinical utility of ^{99m}Tc-glucarate. In particular, studies of myocytes and glial cells will be important before extrapolation of the present results to cardiac and cerebral accumulation of ^{99m}Tc-glucarate.

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