Cellular Uptake Mechanisms of $^{99m}$TcN-NOET in Cardiomyocytes From Newborn Rats

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Background—$\text{Bis[N-ethoxy,N-ethyl(dithiocarbamato)]nitrido Tc (V)}$ (TcN-NOET) is a new technetium complex proposed as a tracer of myocardial perfusion. However, its cellular uptake mechanisms are unknown, although membrane localization on rat heart preparations and preferential binding to polymorphonuclear neutrophils (PMNs) have been reported. Because of the central role of calcium in PMN actions, a relationship was hypothesized between this ion flux and TcN-NOET cellular uptake.

Methods and Results—The mechanisms of cellular uptake of TcN-NOET were investigated in newborn rat cardiomyocytes by study of the effect of calcium channel modulators on tracer binding. Nifedipine had no effect on tracer uptake at 1 minute. However, verapamil 0.1 μmol/L and diltiazem 0.5 μmol/L induced a 40% decrease in uptake. Conversely, Bay K 8644 0.25 μmol/L increased TcN-NOET uptake by 73%. Alterations in other membrane ion transports failed to modify tracer uptake, indicating the specificity of the relationship between TcN-NOET uptake and calcium channels. Kinetic studies indicated that cellular net accumulation of the tracer was slow ($t_{1/2}=28.5$ minutes) and retention was prolonged (84% of initial activity retained after 120 minutes of washout). The energy dependence of TcN-NOET uptake was investigated after 60 minutes of metabolic inhibition by iodoacetic acid plus rotenone. The ATP decrease was not associated with reduction in tracer uptake at 1 minute ($114.9\pm21.9\%$ of control, $P=\text{NS}$).

Conclusions—The decrease in uptake observed with verapamil and diltiazem, the increase with Bay K 8644, and the lack of effect with nifedipine suggest that TcN-NOET binds to L-type calcium channels in the open configuration, without entering cardiomyocytes. The kinetics of TcN-NOET accumulation and retention are slow, and the mechanism for cellular uptake is not energy-dependent. From a clinical point of view, the effect of concurrent treatment by calcium inhibitors on myocardial binding of TcN-NOET should be taken into account. (Circulation. 1998;98:2591-2597.)

Key Words: technetium ■ myocytes ■ calcium channels
beled perfusion tracers.15–17 Thus, cellular uptake of TcN-NOET was studied on newborn rat cardiomyocytes in the presence of modulators of L-type calcium channels and other drugs that inhibit specific membrane ion transports. Cellular accumulation and retention kinetics were also conducted, as well as studies on the influence of the energy state of the cell on cellular uptake of the tracer.

**Methods**

**Cell Culture**

Monolayer cultures of cardiomyocytes from newborn rats were prepared according to the method described by Harary and Farley,18 as modified by Blondel et al.19 The cells (10⁶ cells/mL in 35-mm Petri dishes) were maintained in 95% air/5% CO₂ for 3 days.

**Preparation of TcN-NOET**

TcN-NOET was synthesized as previously described20 with a kit donated by Cis Bio International (France). The solubilizer dimethyl-β-cyclodextrin was not used because the concentration of tracer (10⁻¹² mol/L) did not pose any problem of solubilization. The radiochemical purity, checked by silica thin-layer chromatography (TLC) with dichloromethane as the mobile phase, exceeded 95%. The complex remained stable for ≥6 hours.

**Solutions**

The composition of the cell culture medium was as follows: Ham’s F10 medium, 80% (vol/vol); FCS, 20% (vol/vol); penicillin-streptomycin 0.1% (vol/vol); CaCl₂·2H₂O 1 mmol/L; pH 7.4. Thirty minutes before the addition of the tracer, the culture medium was replaced by the same medium free of FCS. Potassium cyanide (KCN), tetraethylammonium chloride (TEA), ouabain, diflazem, and TcN-NOET were prepared in a physiological medium containing (mmol/L): NaCl 127, KCl 5.4, KH₂PO₄ 1.1, Na₂HPO₄ · 2H₂O 1.1, glucose 6.1, at pH 7.4. Trypsin (1 g · L⁻¹, pH 7.6) was made up in the same solution. Rotenone, isothiocyanic acid (IAA), amiloride, bumetanide, disopyramide, A 23187, verapamil, and nifedipine were dissolved in DMSO, the final concentration of which was always <0.5% (vol/vol). Bay K 8644 had to be dissolved with this solvent at 1.4% (vol/vol).

To ensure that TcN-NOET was maintained in a free form after incubation in the different media or drugs used in the experiments, controls were performed with TLC to measure the migration rate (R₀) of the tracer.

**Uptake and Retention of TcN-NOET**

The TcN-NOET solution was prepared to obtain a final concentration of 10⁻¹² mol/L by addition of 1 μCi (37 kBq) of TcN-NOET (2 mCi/mL and 0.5 Ci/mmol) in 2 mL of culture medium.

Uptake and retention experiments were initiated by adding the tracer to each dish. Aliquots of the loading buffer were obtained to normalize the cellular uptake values to the extracellular concentration of TcN-NOET.

Tracer uptake was stopped by quickly rinsing the cells 3 times with ice-cold buffer solution and adding 2 mL per dish of a 1% SDS solution containing 10 mmol/L sodium borate. The cellular protein content was determined in these samples according to the method of Lowry.20 Radioactivity was assayed with a gamma scintillation detector (Novelec). Knowledge of the elution history of the generator allowed the determination of the quantity and absolute concentration of TcN-NOET in the solutions used. Tracer uptake values were then expressed in femtomoles of cellular TcN-NOET per milligram of proteins normalized to the extracellular concentration of TcN-NOET (fmol · mg protein⁻¹ · mmol/L⁻¹) (Figure 1).

To demonstrate a potential relationship between calcium transport and TcN-NOET uptake, cardiomyocytes were incubated for 15 minutes with L-type calcium channel inhibitors: nifedipine 0.5, 1, and 10 μmol/L, verapamil 0.01, 0.1, 0.5, and 1 μmol/L, and diltiazem 0.1, 0.2, 0.5, 5, and 10 μmol/L, or with an activator of these channels, Bay K 8644, at 0.1, 0.25, 1, and 10 μmol/L. The calcium ionophore A 23187 10⁻⁷ mol/L, amiloride 100 μmol/L (inhibitor of Na/Ca and Na/H exchange), ouabain 100 μmol/L (inhibitor of Na,K-ATPase), bumetanide 10 μmol/L (inhibitor of Na/K/2Cl cotransport), TEA 100 μmol/L (inhibitor of potassium channels), and disopyramide 100 μmol/L (inhibitor of sodium channels) were also tested. These concentrations were those generally used to demonstrate tracer uptake mechanisms.17,21,22 Uptake of TcN-NOET was measured for 1 minute.

Net accumulation of the tracer was measured after incubation periods of 1, 5, 15, 30, 45, 60, 70, 80, 90, 120, or 180 minutes.

Washout kinetics were studied after preincubation with the tracer for 30 minutes, before the cells were rinsed and an isotopically-free solution was again added for periods of 0, 5, 15, 30, 60, or 120 minutes. In some experiments, this solution contained verapamil, diltiazem, or nifedipine at a concentration of 0.5, 10, and 1 μmol/L, respectively. Elution for 120 minutes in the presence of BSA at various concentrations was also performed.

To determine the effect of metabolic inhibition on TcN-NOET uptake, other cells were preincubated for 60, 90, 150, or 180 minutes with 10 μmol/L KCN, 10 μmol/L rotenone, 100 μmol/L IAA, or IAA plus rotenone. Intracellular ATP content and LDH release were measured at each preincubation time. Tracer uptake at 1 minute was measured after 60 minutes of preincubation with the different metabolic inhibitors.

**Analytical Measurements**

Cellular ATP was extracted as previously described.21 The sample was assessed for radioactivity and frozen to allow subsequent ATP measurements by high-performance liquid chromatography at 254 nm with a column of the inverse-phase type (Waters μBondapak C18, 3.9×300 mm). The eluent was a solution of sodium pyrophosphate 0.01 mol/L, 95% methanol 5%, at pH 6. Standards showed good reproducibility of measurements. Cell ATP content was expressed in mmol · (mg protein)⁻¹.

LDH release was measured according to the method of Wroblewsky and La Due23 with a standard Boehringer LDH-P kit and an Eppendorff PCP 6121 robot. Total cellular LDH was measured in preparations disrupted in distilled water. LDH release (U/mL) was normalized to milligrams of cell protein and expressed as a percentage of total LDH.

**Materials**

DMSO, ATP, BSA, and all drugs except KCN were obtained from Sigma Aldrich Chimie SARL; Ham’s F10 medium and penicillin (10 000 U/mL)–streptomycin (10 mg/mL) from Techgen International; KCN and TLC silica gel plates from Merck-Clevon SA; trypsin from DIFCO Laboratories; and FCS from Gibco BRL.
Statistical Analysis

Results were expressed as mean±SD. Statistical significance was determined by Student’s t test for nonpaired series. The significance threshold was fixed at $P<0.05$.

Results

Stability and Interaction of TcN-NOET With the Drugs and Media Used

None of the drugs used, apart from KCN, altered the TcN-NOET chemical entity in vitro. With KCN, the technetium atom is separated from its ligand. KCN was thus removed before the addition of the tracer.

The $R_f$ of TcN-NOET was always 0.9, irrespective of conditions (data not shown), indicating that TcN-NOET remained in a free form. The only binding sites for TcN-NOET were therefore cellular.

Control experiments showed that DMSO 1.4% (vol/vol) had no effect on cellular uptake of TcN-NOET.

Relationship Between Uptake and Retention of TcN-NOET and Calcium Channels

Uptake kinetics were determined for a tracer concentration of $1.36±0.09\times10^{-12}$ mol/L (ie, $2.72±0.18\times10^{-15}$ mol/dish). Net accumulation by cardiomyocytes reached a steady state after a 90-minute incubation period, with maximum uptake representing 59% of the injected dose. The time required to reach half of maximum uptake ($t_{1/2}$) was 28.5 minutes.

Nifedipine had no effect on TcN-NOET uptake at any of the doses tested (Figure 2A). However, verapamil 0.1 μmol/L and diltiazem 0.5 μmol/L caused a marked reduction of 30% to 40% in TcN-NOET binding (Figures 2B and 2C). Bay K 8644 induced an increase in tracer uptake of 40%, 75%, 50%, and 40% at concentrations of 0.1, 0.25, 1, and 10 μmol/L, respectively (Figure 2D). The calcium ionophore A 23187 and the other ion transport inhibitors had no effect on TcN-NOET uptake (Figure 3).

Washout kinetics indicate that, after a 120-minute rinsing period, the TcN-NOET activity bound to cells represented...
84% of the initial activity. This retention was not modified by verapamil, diltiazem, or nifedipine (data not shown).

On the other hand, cellular retention of TcN-NOET after 120 minutes of washout decreased in a dose-dependent manner with BSA concentrations of 0.17, 0.33, 0.50, 0.75, 1, and 1.5 mg/mL: this retention was 96%, 89%, 82%, 64%, 45% and 35%, respectively, of the retention observed without BSA (Figure 4).

Uptake of TcN-NOET and Metabolic Inhibitors
To validate the metabolic inhibition model, the ATP content of cells and LDH release by cardiomyocytes were determined according to the type and duration of inhibition (Figure 5).

With a 60-minute incubation period, the various metabolic inhibitors permitted different intracellular ATP levels to be obtained without damaging the cells, as evidenced by LDH release. When intracellular ATP content was intact, uptake at 1 minute was increased by 136% with KCN (P<0.001) and 38% with rotenone (P<0.05). When intracellular ATP content was decreased by 47% (with IAA), 1-minute uptake was increased by 78% (P<0.001). However, when ATP was totally depleted (with IAA plus rotenone), uptake remained unchanged (Table).

Discussion
TcN-NOET is a new tracer of myocardial perfusion. Its macroscopic behavior in dogs and humans has been well characterized, and its clinical use can now be envisaged. However, its detailed cellular binding mechanisms are unknown. Current data indicate that TcN-NOET binds to cell membranes. The observations made by Moisan et al, whereby TcN-NOET binds preferentially to neutrophils in the presence of whole blood, suggest that there is a structure in the PMN membranes that causes the tracer to bind to this cell type. Given the preponderant role of calcium in neutrophil function, newborn rat cardiomyocytes were used to determine

ATP Content and 1-min TcN-NOET Uptake After 60-min Metabolic Inhibition

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>ATP Content, nmol/mg Cell Protein</th>
<th>1-min TcN-NOET Uptake, % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.1±4.4</td>
<td>100.00±11.72</td>
</tr>
<tr>
<td>KCN 10 μmol/L</td>
<td>15.3±0.9</td>
<td>236.98±56.02†</td>
</tr>
<tr>
<td>Rotenone 10 μmol/L</td>
<td>14.2±3.6</td>
<td>138.69±25.30*</td>
</tr>
<tr>
<td>IAA 100 μmol/L</td>
<td>7.9±0.9†</td>
<td>178.00±25.90†</td>
</tr>
<tr>
<td>IAA+rotenone</td>
<td>0.9±0.1†</td>
<td>114.89±21.94</td>
</tr>
</tbody>
</table>

ATP content and 1-min TcN-NOET uptake were determined in the same preparations.

*P<0.05, †P<0.001 vs control.
whether a modification of calcium fluxes could influence cellular uptake of TcN-NOET.

**Uptake and Retention of TcN-NOET Relationship With Calcium Channels**

TcN-NOET net accumulation reached a plateau after a 90-minute incubation period, with a \( t_{1/2} \) of 28.5 minutes. This value is similar to that of Tc-MIBI (35 minutes) and significantly longer than that of \(^{203}\text{TI} \) (4.95±0.51 minutes) and teboroxime (<2 minutes). However, this similarity between TcN-NOET and Tc-MIBI uptake does not mean that their cellular uptake mechanisms are identical. Intracellular accumulation of Tc-MIBI is made possible essentially through its positive charge, allowing its sequestration within mitochondria after diffusion through the membranes because of its lipophilic nature. This mechanism cannot be envisaged for TcN-NOET, which is a neutral molecule. The rapid extraction of \(^{203}\text{TI} \) is because its entry into the cells is specifically via Na,K-ATPase. The fact that the \( t_{1/2} \) of uptake of teboroxime, which is distributed nonspecifically in the cell membranes, is lower than that of TcN-NOET may be due to the greater lipophilicity of teboroxime (partition coefficient octanol/water, 40 000 [Reference 25] compared with 3100 for TcN-NOET, unpublished data), which could result in faster distribution between the aqueous culture medium and the cell membranes.

Verapamil and diltiazem decreased TcN-NOET uptake by 40%. The dihydropyridine nifedipine had no effect, whereas an activator of the same class, Bay K 8644, increased TcN-NOET uptake by 70%. Verapamil and diltiazem. For the remaining 60% of cellular uptake of TcN-NOET, this relationship would involve 40% of total cellular uptake of TcN-NOET. Binding would take place on the calcium channel in an open configuration. Conversely, all of the experimental data can be explained by binding of TcN-NOET to the channel protein in its open configuration.

The path of access of verapamil and diltiazem (phenylalkylamine and benzothiazepine family, respectively) to their binding site is the lumen of the channel. By contrast, nifedipine, which is neutral and lipophilic, can access its binding site through either the channel opening or the lipid bilayer. Furthermore, nifedipine acts allosterically at a distance from its binding site, whereas verapamil interferes presumably directly with calcium ion fluxes. The lack of effect of nifedipine on TcN-NOET uptake and the fact that TcN-NOET is a neutral complex do not argue in favor of cellular entry of TcN-NOET via calcium channels. Conversely, all of the experimental data can be explained by binding of TcN-NOET to the channel protein in its open configuration.

The addition of verapamil, diltiazem, or nifedipine to the rinsing solution did not accelerate washout of the molecule. This result is not contrary to the hypothesis that TcN-NOET binds to the calcium channel in its open configuration but indicates that the binding sites for TcN-NOET and the 3 calcium inhibitors are different.

Cellular retention of TcN-NOET decreased after 120 minutes of elution with a medium supplemented with BSA, this effect being dose-dependent. These results suggest that addition of BSA led to the introduction of nonspecific binding sites into the elution solution, thereby allowing nonspecific distribution of TcN-NOET between cell membranes and culture medium. Without BSA, the aqueous nature of the culture medium promoted retention of the tracer in the membrane. These results can be compared with those obtained on isolated perfused rat hearts, which show that myocardial retention of TcN-NOET was decreased only when blood elements were added to the perfusion medium.

The results obtained to date indicate the existence of a relationship between cellular binding of TcN-NOET and calcium channels. On the basis of the maximum effect obtained with verapamil and diltiazem, this relationship would involve 40% of total cellular uptake of TcN-NOET. Binding would take place on the calcium channel in an open configuration at a different binding site from that of nifedipine, verapamil, or diltiazem. For the remaining 60% of cellular uptake, TcN-NOET would be distributed nonspecifically between the cell membranes and the proteins present in the extracellular medium.

**Uptake of TcN-NOET: Effect of Metabolic Inhibitors**

Although KCN is a known metabolic inhibitor, it had no effect on ATP at the concentration used (10 \( \mu \)mol/L). Similar results were observed by others for cyanide concentrations of 1 mmol/L. This drug abolishes oxygen consumption, but the cells compensate for the ATP deficit of mitochondrial origin through an increase in glycolysis.

Rotenone had no effect on the ATP content of cardiomyocytes. First, although this drug blocks complex I of the mitochondrial electron chain, the reduced cofactor FADH\(_2\) could be oxidized downstream of this complex, allowing the necessary proton gradient for ATP synthesis to be maintained. Second, cardiomyocytes would be able to compensate for the reduced energy production by increasing anaerobic glycolysis.

IAA progressively depletes cells of ATP by inhibiting anaerobic glycolysis.

Finally, incubation of cardiomyocytes with IAA plus rotenone led to total depletion of ATP as early as the 60th minute of incubation. In this case, IAA prevented any increase in anaerobic glycolysis secondary to the inhibition of the respiratory chain by rotenone, and energy production was no longer possible.

When metabolic inhibition lasted <90 minutes, energy depletion was not associated with significant LDH release.
60-minute incubation period with the different metabolic inhibitors was therefore chosen for the study.

For a negligible amount of intracellular ATP (IAA plus rotenone), uptake of TcN-NOET remain unchanged, whereas for an intermediate ATP amount (IAA), uptake was increased. These results indicate that a reduction in intracellular ATP was not associated with a reduction in tracer binding. Furthermore, the increase in uptake at 1 minute, which reached 136% and 38% with KCN and rotenone, respectively, took place at an intracellular ATP content that did not differ significantly from that of controls. This increase can therefore only be the result of a particular effect of these drugs. The effect of cyanide could be explained by the alterations it induces on transmembrane calcium movements. Indeed, cyanide induces an increase in intracellular calcium alterations it induces on transmembrane calcium movements. Therefore only be the result of a particular effect of these L-type calcium channels. The increase observed with IAA and rotenone remains to be elucidated, especially because uptake of TcN-NOET was not changed when these 2 inhibitors were used together.

Clinical Implications

In clinical practice, the blood concentrations obtained during treatment with calcium inhibitors are within the range of 10⁻⁷ mol/L. A reduction in myocardial uptake of TcN-NOET in humans taking calcium channel blockers can thus be envisaged, which would be detrimental to the quality of the images obtained and could thus lead to clinical misinterpretation.

A further finding is that a reduction in intracellular ATP does not lead to a reduction in TcN-NOET uptake. Unlike ²⁰¹Tl or Tc-MIBI, TcN-NOET is not a tracer of “cellular viability,” but this does not mean that this complex cannot be used as a tracer of “myocardial viability” in clinical practice. The cell system is a pure flow model with a relatively high and steady input function that does not correspond to in vivo kinetics. TcN-NOET redistributes in dogs and humans, so that there is likely to be a metabolic component involved with differential uptake and washout. Further experiments are necessary to help understand this phenomenon.

In summary, on newborn rat cardiomyocytes, the kinetics of TcN-NOET uptake were slow and not energy-dependent. The working hypothesis, namely, that there is a relationship between cellular binding of TcN-NOET and calcium movements, would seem to have been confirmed for this cell type. Indeed, the activation or inhibition of L-type calcium channels affects tracer binding. It seems that TcN-NOET does not enter into cardiomyocytes but binds to plasma membranes with a greater affinity for L-type calcium channels in an open configuration than other membrane proteins. This specific binding to L-type calcium channels in our model does not exclude specific binding to other types of calcium channel. Indeed, TcN-NOET binds preferentially to PMNs when incubated with whole blood, whereas calcium movements for this cell type do not take place through L-type calcium channels.

These results must be taken into account if TcN-NOET is used in clinical practice. The action of calcium inhibitor treatments on myocardial uptake of TcN-NOET may have an effect on the quality of the scintigraphic images and thus on the clinical interpretation of these images.

References


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