Two Sodium/Calcium Exchanger Gene Products, NCX1 and NCX3, Play a Major Role in the Development of Permanent Focal Cerebral Ischemia

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Background and Purpose—The Na⁺/Ca²⁺ exchanger, by mediating Ca²⁺ and Na⁺ fluxes in a bidirectional way across the synaptic plasma membrane, may play a pivotal role in the events leading to anoxic damage. In the brain, there are 3 different genes coding for 3 different proteins: NCX1, NCX2, and NCX3. The aim of this study was to determine whether NCX1, NCX2, and NCX3 might play a differential role in the development of cerebral injury induced by permanent middle cerebral artery occlusion (pMCAO).

Methods—By means of Western blotting, NCX1, NCX2, and NCX3 protein expression was evaluated in the ischemic core and in the remaining nonischemic area of the slice at different time intervals starting from ischemia induction. The role of each isoform was also assessed with antisense oligodeoxynucleotides (ODNs) targeted for each isoform. These ODNs were continuously intracerebroventricularly infused with an osmotic minipump (1 μL/h) for 48 hours, 24 hours before pMCAO.

Results—The results showed that after pMCAO all 3 NCX proteins were downregulated in ischemic core; NCX3 decreased in perinfracellular area whereas NCX1 and NCX2 were unchanged. The ODNs for NCX1 and NCX3 gene products were capable of inducing an increase in the ischemic lesion and to worsen neurological scores.

Conclusions—The results of this study suggest that in the neuroprotective effect exerted by NCX during ischemic injury, the major role is prevalently exerted by NCX1 and NCX3 gene products. (Stroke. 2004;35:000-000.)

Key Words: neuroprotection ■ oligodeoxynucleotides, antisense ■ protein expression ■ sodium–calcium exchange

The Na⁺/Ca²⁺ exchanger (NCX) consists of 9 transmembrane segments that can mediate Ca²⁺ and Na⁺ fluxes across the plasma membrane.¹

Three different genes, ncx1, ncx2, and ncx3, coding for 3 different proteins have been cloned.²⁻⁴ Unlike other organs and tissues, in the brain all 3 proteins are present.⁵ These 3 proteins are differentially expressed in distinct regions of the central nervous system where they might underlie different physiological and pathophysiological functions.⁶⁻⁸

Depending on the intracellular concentrations of Ca²⁺, [Ca²⁺]i, and Na⁺, [Na⁺], NCX can operate either in the forward mode, coupling the uphill extrusion of Ca²⁺ to the influx of Na⁺ ions, or in the reverse mode, mediating the extrusion of Na⁺ and the influx of the Ca²⁺ ions.¹ During ischemia, a compromise of the 2 plasma membrane ATP-dependent pumps, Na⁺/K⁺ ATPase and Ca²⁺ ATPase, occurs with a consequent derangement of [Na⁺]i and [Ca²⁺]i.¹ Thus, by controlling intracellular homeostasis of these 2 ions, NCX may play a pivotal role in the events leading to anoxic damage. Although a great number of conflicting reports¹⁰⁻¹⁴ have been published on the role played by NCX during focal ischemia, it has recently been demonstrated that, in in vitro and in vivo models of anoxia and ischemia, a stimulation of NCX activity may help neurons and glial cells to survive, whereas its pharmacological blockade can compromise their survival.¹¹,¹²,¹⁴

Information on the pattern of expression and on the role of each specific NCX gene product in those brain regions damaged during focal ischemia is not available. To address this question, NCX1, NCX2, and NCX3 protein expression was investigated by Western blot analysis at different times in the ischemic core and in the remaining nonischemic area of the slice of rats bearing permanent middle cerebral artery occlusion (pMCAO). Furthermore, knocking out each NCX gene product through antisense oligodeoxynucleotides (AS-ODNs) was used to evaluate the specific role played by each NCX protein in the process of neuronal damage during ischemic insult.

Materials and Methods

Experimental Groups

Male Sprague-Dawley rats (Charles River, Italy) weighing 250 to 270 g were housed under diurnal lighting conditions (12 hours...
Experiments were performed on 161 rats according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the "Federico II" University of Naples.

Surgical Procedures

All rats, anesthetized with chloral hydrate (400 mg/kg, IP), were put on a stereotaxic frame. An Alzet osmotic minipump (model 1003D, delivery rate 1 μL/hr) was inserted into a subcutaneous pouch and connected through a plastic catheter to the brain infusion cannula placed into the right lateral ventricle. Twenty-four hours after cannula implantation, pMCAO was induced as previously described. A 2-cm incision was made vertically between the orbit and the ear. Under an operating stereomicroscope (Nikon SMZ800), an incision was made to divide the temporal muscle. The left lateral aspect of the skull was then exposed by reflecting the temporal muscle surrounding the soft tissue. A small window was made just over the visibly identified middle cerebral artery. Saline solution was applied to the area throughout the procedure to prevent heat injury. The left MCAO was performed by electrocoagulation with a bipolar electrocauterizer (Diatemo MB122; GIMA). pMCAO was performed as close as possible to its origin, near the circle of Willis. The body temperature was continuously monitored with a rectal probe (Homoeothermic Blanket System, Harvard Apparatus) and maintained at 37±0.5°C until the end of the surgical procedure. AS-ODNs did not affect body temperature.

Monitoring of Blood Gas Concentration and Cerebral Blood Flow With Laser-Doppler Flowmetry

In some of the experimental and control rats, a catheter was inserted into the femoral artery to measure arterial blood gases with a blood gas analyzer before and after ischemia (Rapid Laboratory 860, Chiron Diagnostic). Cerebral blood flow was monitored in the cerebral cortex ipsilateral to the occluded MCA. AS-ODNs treatment did not affect the cerebral blood flow, PaO₂, PaCO₂, and pH mean values both in lesioned and sham-operated animals (Table 1).

Evaluation of the Ischemic Volume and of Neurological Deficit Scores

Rats were decapitated 24 hours after ischemia at which time the extension of infarct is maximal. The ischemic volume was evaluated by 2,3,5 triphenyl tetrazolium chloride (TTC) staining. The brains were quickly removed and placed in ice-cold saline and then cut into 500-μm coronal slices with a vibratome (Campden Instrument, 752 mol/L), beginning 0.5 mm posterior to the anterior pole. Sections were incubated in 2% TTC containing saline solution for 20 minutes and in 10% formalin overnight. The infarction area, outlined in white and manually defined by an electronic pencil on the display screen, was automatically calculated with image analysis software (Image-Pro Plus 4.1).

The total volume of the lesion was calculated by summing the infarct area in each section and by multiplying it by the distance between sections (12 sequential 500-μm rostro-caudal sections). Edema was calculated as follows: volume of the hemisphere ipsilateral to the lesion—volume of the hemisphere contralateral to the lesion. This value was expressed as percentage of the volume of the hemisphere ipsilateral to the lesion (volume of edema: volume of hemisphere ipsilateral to the lesion x 100). This percentage was subtracted from the volume of the infarct. The person who did the image analysis was blinded to the study groups.

Figure 1. Specificity of antibodies against NCX1, NCX2, or NCX3. When the antibody against NCX1 was tested in BHK cells wild-type or transfected with NCX1, NCX2, or NCX3, it reacted only with the specific NCX1 protein, whereas it did not react with the other 2 isoforms (A). Analogously, the antibodies against NCX2 or NCX3 were able to react with the specific isoform only in BHK cells transfected with NCX2 or NCX3 cDNAs, respectively (B and C).

Figure 2. Time course of NCX1, NCX2, and NCX3 protein expression after pMCAO in area 1 and in area 2. Western blot and its densitometric analysis of NCX1, NCX2, and NCX3 proteins in area 1 (A) and in area 2 (B) are represented. Data were normalized on the basis of β-actin levels and expressed as percentage of sham-operated controls (CTL). Values were mean±SEM (n=8 in panel A and n=11 in panel B). *P<0.05, compared with CTL.
In all animals, 24 hours after ischemia, neurological function was scored according to 2 scales: a general neurological scale (general score) and a focal neurological scale (focal score), validated in rats by Matrone et al. In the general score, these 6 general deficits were measured: (a) hair conditions (0–2), (b) position of ears (0–2), (c) eyes conditions (0–4), (d) posture (0–4), (e) spontaneous activity (0–4), and (f) epileptic behavior (0–12). For each of the 6 general deficits measured, animals received a score depending on the severity of the symptoms. The scores of investigated items were then summed to provide a total general score ranging from 0 to 28. In the focal score, these 7 areas were assessed: (a) body symmetry, (b) gait, (c) climbing, (d) circling behavior, (e) front limb symmetry, (f) compulsive circling, and (g) whisker response. For each of these items, items were rated between 0 to 4 depending on the severity. The 7 items were then summed to give a total focal score ranging between 0 to 28. AS-ODN treatment did not induce any change of neurobehavioral scores in sham-operated rats (data not shown).

**Experimental Protocol**

Chimeric phosphorothioated antisense, AS1- (GenBank Accession Number X68191 S53347), AS2- (GenBank Accession Number U08141), and AS3- (GenBank Accession Number NM078620) ODNs or sense (S1-, S2-, and S3-) ODNs (Primm, Italy) were designed to target the area near the start region (italicized bases are phosphorothioated): AS1 (–264 to 10): 5'-TGGACTTCCAATTTGT-3' S1: 5'-AAAGTTGAAAGCTCCA-3' AS2 (+61/+73): 5'-TCGGAGTAGCTTC-3' S2: 5'-GAAGCTTCCCGA-3' AS3 (–9/+5): 5'-GCCATACACAAAG-3' S3: 5'-CTCTTTGTTGTAGCC-3' Phosphorothioated ODNs display an increased stability and are more easily diffused into the whole brain when they are ICV infused. Antisense or sense ODNs were continuously intracerebroventricularly (ICV) infused (70 μg/kg) with osmotic minipumps for 48 hours, starting 24 hours before pMCAO induction.

**Identification of Ischemic Area**

The brains were removed at various times after pMCAO and placed on ice. Beginning at 1 mm posterior to the anterior pole, 0.5 or 2 mm thick coronal slices were alternatively cut twice. The 0.5 mm slices, stained in 2% TTC-saline solution were used to identify the ischemic core. The 2-mm slices were used to dissect area 1 of Figure 2, identified by TTC in the previous adjacent slice, and to dissect area 2 of Figure 2. The corresponding areas on the contralateral hemisphere were processed with the same procedure. After surgical removal, the samples were immediately frozen in liquid nitrogen and kept at −80°C until analysis.

**Figure 3.** Effect of AS1-, AS2-, and AS3-ODNs on the expression of NCX1, NCX2, and NCX3 proteins, in a region corresponding to the ischemic core and the remaining nonischemic area of the slice located in the contralateral hemisphere. Western blot and its densitometric analysis of NCX1, NCX2, and NCX3 proteins, after the ICV administration of AS1-, AS2-, and AS3-ODNs, in a contralateral area corresponding to a region including the ischemic core and the remaining nonischemic area of the slice. Data were normalized on the basis of β-actin levels and represented as percent of the expression of each NCX isoform in vehicle-injected rats bearing pMCAO. Values are mean±SEM (n=3), *P<0.05 vs respective isoform levels in vehicle-injected rats bearing pMCAO.

**Protein Expression Analysis**

Total protein extracts from dissected areas of ischemic and nonischemic brains were obtained as previously described by Matrone et al. Protein concentration was determined using the Bradford method. Protein samples (50 μg) were analyzed on 8% sodium dodecyl sulfate polyacrylamide gel with 5% sodium dodecyl sulfate stacking gel (SDS-PAGE) and electrottransferred onto Hybond ECL nitrocellulose paper (Amersham). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 (TBST; 2 mmol/L Tris-HCl, pH 7.5) for 2 hours at room temperature and subsequently incubated overnight at 4°C in the blocked buffer with the 1:500 antibody for NCX1 (monoclonal mouse antibody, Swant), 1:200 antibody for NCX2 (polycyonal rabbit antibody, Alpha Diagnostic), and 1:200 antibody for NCX3 (polycyonal rabbit antibody, Alpha Diagnostic). The specificity of the 3 antibodies was tested in baby hamster kidney (BHK) cells wild-type or transfected with NCX1, NCX2, or NCX3 cDNAs.

The membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 hour (Amersham). Immunoreactive bands were detected with the ECL (Amersham). The optical density of the bands (normalized with that of β-actin) was determined by Chemi Doc Imaging System (Biorad).

**Statistical Analysis**

Values are expressed as mean±SE. Statistical analysis was performed with ANOVA followed by Newman-Keuls test. Statistical significance was accepted at the 95% confidence level (P<0.05).

**Figure 4.** Effect of AS1-, AS2-, and AS3-ODNs on infarct volume induced by pMCAO. Each column represents the mean±SE of the ischemic volume. Each AS- or S-ODN (70 μg/kg) was continuously infused ICV by an osmotic minipump (1 μL/h) 24 hours before and after pMCAO. Control rats received vehicle. Ischemic rats were euthanized 24 hours after pMCAO. Each experimental group consisted of 7 animals. *P<0.05 vs vehicle-treated ischemic rats and respective S-ODN group.
Results

Specificity of Antibodies Against NCX1, or NCX2, or NCX3
Each antibody reacted only in the BHK clone expressing the corresponding specific NCX protein, whereas it was inactive in the other BHK clones (Figure 1).

Time Course of NCX1, NCX2, and NCX3 Protein Expression in Area 1 and in Area 2 After pMCAO
In area 1, a 50% reduction of all 3 proteins occurred at 6 and 24 hours after pMCAO, whereas at 72 hours NCX1 and NCX3 proteins almost disappeared, although NCX2 protein expression continued to be halved (Figure 2A). In area 2, NCX3 showed a 60% reduction 6 to 72 hours after pMCAO, whereas NCX1 and NCX2 did not change (Figure 2B). In the brain regions of the contralateral hemisphere corresponding to area 1 and 2, NCX1, NCX2, and NCX3 protein levels did not change as compared with the values obtained in the ipsilateral hemisphere of the sham-operated animals (data not shown).

Effect of AS1-, AS2-, and AS3-ODNs on Infarct Volume and on Neurological Scores Induced by pMCAO
Each specific NCX1, NCX2, or NCX3 AS-ODN produced a reduction in the respective gene product in a region consisting of area 1 plus area 2 (data not shown) and in the corresponding region of the intact contralateral hemisphere 48 hours after ICV AS-ODNs infusion (Figure 3). Each AS-ODN did not affect the expression of the other 2 NCX isoforms (Figure 3). In addition, each AS-ODN was able to prevent Na+/Ca2+ exchanger (NCX) isoforms from achieving the ischemic volume, whereas AS2-ODN did not change the infarct size (Figure 4). Edema volumes in AS1-ODN (4.64±3.11), AS2-ODN (6.15±3.26), and AS3-ODN (6.74±3.85) were not significantly different from those of S1-ODN (5.10±3.24), S2-ODN (6.2±5.4), S3-ODN (4.85±2.75), and vehicle-treated (4.5±3.7) rats. The 3 AS-ODNs were devoid of direct neurotoxic effects, because they did not cause any brain damage in normal rats.

The infarct enlargement induced by AS1- and AS3-ODNs was associated with a worsening of focal and general neurological deficits (Figure 5).

Discussion
The results of this study demonstrated for the first time that pMCAO caused relevant and selective changes in the pattern of NCX protein expression in area 1 and in area 2. In these 2 areas, all 3 NCX proteins have been shown to be expressed.8 Particularly, in area 1, the results of the present study showed that 6 to 72 hours after pMCAO a significant decrease in NCX1, NCX2, and NCX3 occurred. These findings could be attributed to the cleavage of NCX proteins by proteolytic enzymes activated during ischemic injury, such as calpain and caspases. In fact, it has been recently shown that NCX and the other calcium transporters, Ca2+/ATPase, can be cleaved by caspase and calpain.25 The change of the expression in NCX1, NCX2, and NCX3 gene products after pMCAO seems to suggest that these isoforms can be involved in the process leading to cell death or survival in those areas that are interested by the ischemic insult.

This hypothesis is sustained by the results showing that in knocking down 2 of these proteins, NCX1 and NCX3, with selective AS-ODNs, a remarkable enlargement of the infarct volume occurred. Such a phenomenon was associated with a

### Physiological Blood Parameters Following ODNs Treatment After Ischemia

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Values are mean±SE (n=3 for pH, PaO2, and PaCO2; n=4 for CBF). PaO2 and PaCO2 are expressed in mm Hg. CBF values, evaluated 30 minutes after pMCAO, are expressed as percentage of baseline value. ANOVA followed by Newman-Keuls test revealed no significant intergroup difference for any variable.
worsening of neurological deficits. By contrast, a large reduction of NCX2 protein levels with the specific AS2-ODN failed to induce any change in infarct size. The lack of effect of AS2-ODN suggests that NCX2 does not exert a remarkable role in cell death during ischemic conditions. On the other hand, evidence that NCX exerts a neuroprotective action causing a reduction of infarct size, whereas its inhibition leads to an enlargement of the lesioned area, has recently been provided. As regards the question how NCX exerts its protective role in brain ischemia, it can be hypothesized that, because in the penumbral region there is still a persistence of ATPase activity, NCX would be operating in a forward mode. In this way, NCX will be extruding Ca++ ions favoring entry of Na+ ions. Therefore, NCX inhibition at this stage would reduce Ca++ ion elimination, thus enhancing Ca++-mediated cell injury. By contrast, in the ischemic core region, in which a remarkable ATP loss occurs, there is a massive accumulation of intracellular Na+ ions because of the failure of Na+/K+ ATPase. This intracellular Na+ loading should promote the NCX to operate in the reverse mode. Therefore, NCX inhibition by AS-ODNs in the core region would further worsen the necrotic lesion of the surviving cells as intracellular Na+ loading will increase.

In conclusion, the results of this study suggest that in the neuroprotective effect exerted by NCX during ischemic injury, the major role is prevalently exerted by NCX1 and NCX3 gene products.

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References
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