J. Pharm. Pharmacol. 1998, 50: 1405–1407
Received April 30, 1998
Accepted July 6, 1998

Ultrastructural Localization of Nicorandil in the Heart of Rats

SHUNICHI KITAJIMA, YOKO TSUCHIYA* AND KAZUSHIGE SAKAI*

*Panapharm Laboratories Co. Ltd, Uto, Kumamoto 869-04 and *Central Research Laboratories, Chugai Pharmaceutical Co. Ltd, 3-41-8 Takada, Toshima-ku, Tokyo 171, Japan*

Abstract

To examine the intracellular localization of nicorandil in the heart, [^{14}C]nicorandil and [^{3}H]nicorandil (3 mg kg^{-1}) were given orally to rats.

The maximum concentration (C_{max}) of nicorandil in the myocardium was reached 15 min after the oral dosing. At this time subcellular localization of nicorandil was examined. Nicorandil and its denitrated metabolite, SG-86 were found in mitochondrial fractions and in cytosolic and microsomal fractions of the heart. Electron-microscopic autoradiograms recorded 15 min after oral dosing of 3 mg kg^{-1} [^{3}H]nicorandil to rats also showed the presence of silver grains generated by the radioactive nicorandil or its metabolites in the mitochondria of the heart.

We conclude that nicorandil given orally to rats is distributed in mitochondria of the heart.

Nicorandil (*N*-(2-hydroxyethyl)nicotinamide nitrate ester), an orally efficacious drug for treatment of ischaemic heart disease (Sakai 1989; Kinoshita & Sakai 1990; Krumenacker & Roland 1992), is a K_{ATP}-channel opener with nitrate-like activity (Taira 1987). Investigations have shown that K_{ATP}-channel openers have cardioprotective effects in several animal models (Gross 1993). Recently, Garlid et al (1996) suggested that the mitochondrial K_{ATP} channel is an important intracellular receptor that should be taken into account in considering the pharmacology of K_{ATP}-channel activators in in-vitro experiments. This report has attracted our great interest, because the mitochondrial K_{ATP} channels are involved in the control of mitochondrial volume and energetics (Inoue et al 1991; Paucek et al 1992). The aim of the current study was to examine myocardial subcellular localization of nicorandil given orally to rats.

Materials and Methods

Chemicals

Nicorandil, *N*-(2-hydroxyethyl)nicotinamide nitrate ester, and SG-86, *N*-(2-hydroxyethyl)nicotinamide, were synthesized in the Chugai Organic Chemistry Laboratory. [Carbonyl-^{14}C]nicorandil and [pyridine-5-^{3}H]nicorandil were synthesized by Amersham International plc, Little Chalfont, Buckinghamshire, UK. The specific activities of [^{14}C]nicorandil and [^{3}H]nicorandil were 9·58 MBq mg^{-1} and 4·69 GBq mg^{-1}, respectively, and their radiochemical purity 99 and 98%, respectively. The drugs were dissolved in and diluted with distilled water.

Correspondence: K. Sakai, Central Research Laboratories, Chugai Pharmaceutical Co. Ltd, 3-41-8 Takada, Toshima-ku, Tokyo 171, Japan.

Animals

All experiments were performed under Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Male Sprague–Dawley rats (Charles River Japan, Hino), 8 weeks, were fasted overnight before the experiment, but allowed free access to water.

Determination of nicorandil and its metabolite, SG-86

[^{14}C]Nicorandil (3 mg kg^{-1} containing 28·74 MBq radioactivity) was administered orally to rats and the animals were killed 15, 30 or 60 min later under ether anaesthesia. The hearts were excised, rinsed in ice-cold isotonic saline, blotted with filter paper, weighed, homogenized in 80% ethanol by means of a glass-type homogenizer, and centrifuged at 3000 rev min^{-1} at 4°C for 5 min. The supernatant (200 μL) was evaporated, redissolved in 10% methanol (150 μL), and injected into the high-performance liquid chromatograph (HPLC; LC-Module-1; Water Associates, Milford, MA). To isolate subcellular fractions (for samples taken

15 min after oral dosing only) the heart (0·8 g) was homogenized in cold sucrose buffer (0·25 M, adjusted to pH 7·2 with 10 mM Tris–HCl containing 1 mM EDTA at 25°C; 3·2 mL) on an ice-bath. In accord with the description by Hess & Brand (1974), the 20% homogenate was centrifuged at 2800 rev min^{-1} for 10 min, and the supernatant was centrifuged again at 6800 rev min^{-1} for 10 min to prepare a pellet of the mitochondrial fraction. The mitochondrial pellet was resuspended in 1·15% KCl-25 mM Tris–HCl buffer (pH 7·4; 8 mL). The post-mitochondrial supernatant was centrifuged at 9100 rev min^{-1} for 10 min. After centrifugation of the resultant supernatant at 33 200 rev min^{-1} for 60 min to obtain cytosolic and microsomal fractions, the pellet was resuspended in 1·15% KCl-25 mM Tris–HCl buffer (pH 7·4; 4 mL) and microsomal fractions were prepared. Nicorandil and its main metabolite, SG-86, were determined by HPLC on a 250 mm×4·6 mm i.d. TSK gel ODS 80 column (Tosoh); the mobile phase was 10% methanol at a flow rate of 1 mL min^{-1}. The radioactivity of the eluent was detected by means of a Packard liquid-scintillation counter (Tri Carb 2500TR, Meriden, CT), and converted to ng-equivalents of nicorandil or SG-86 g^{-1} (wet weight) of tissue or mg $protein^{-1}$, based on the specific activity of the dose or concentration given. The protein content of each fraction was assessed by the method of Lowry et al (1951), with bovine serum albumin as standard for the calibration plot.

Observation by electron microscopic autoradiography

[^{3}H]nicorandil (3 mg kg^{-1} containing 185 MBq radioactivity) dissolved in distilled water (3 mL kg^{-1}), was given orally to rats. The rats were killed 15 min later and the hearts were removed and cut into small pieces by cross-sectioning the left ventricle, based on the anterior descending branch of the left coronary artery. The radioactive sources were fixed with 2·5% glutaraldehyde dissolved in 0·1 M phosphate buffer, and the tissue was cut into 100-μm slices by means of a DTK-3000W Microslicer (D.S.K., Kyoto, Japan). Thereafter, the sources were fixed again with 2% OsO_4, and embedded in Epon after dehydration. Embedding was performed by the sandwich method using Aclar Film (Nissin E. M., Tokyo, Japan), in accord with the report by Kingsley & Cole (1988). For electron-microscopic autoradiography sections of the radioactive sources were transferred to collodion-coated copper-grids. The grids were held on Grid-sticks (Micro Star Corporation, Tokyo, Japan) and the Grid-sticks were stained with uranyl acetate, covered with a carbon layer approximately 5 nm thick, and then dipped for 10 s at 45°C in Konica's autoradiographic emulsion NR-H2 diluted 1 : 12 with demineralized water. Exposure for 1 month was performed at 4°C in light-tight boxes containing silica gel. The autoradiographs were developed at 20°C for 7 min in Konica Konidor-X, and fixed in Konica Konifix for 15 min at 20°C. After fixation the Grid-sticks were stained with lead citrate. The grids were carefully removed from the Grid-stick and viewed in a Philips EM-400 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 80 kV.

Results and Discussion

Table 1 shows the amounts of nicorandil and its metabolites (SG-86 and unknown substances) in the myocardium during a period 60 min after oral dosing of 3 mg kg^{-1} [^{14}C]nicorandil to rats. The maximum concentration (C_{max}) of nicorandil was reached 15 min after dosing; thereafter the concentration of nicorandil decreased gradually whereas that of its metabolites increased progressively. The subcellular distribution of nicorandil in the heart 15 min after the oral dosing is shown in Table 2. The parent drug, nicorandil, and its metabolite, SG-86, were detected in all subcellular fractions tested, their levels increasing in the order: cytosol > mitochondria > microsome. The electron-microscopic autoradiogram of the left ventricular myocardium clearly demonstrated that the developed silver grains are localized in the mitochondria and, to a lesser extent, in the myofibrils (Figure 1). The mitochondria, having cristae, were spherical and double-layered. Numerous glycogen granules were observed in the myofibrils. Thus it seems that considerable amounts of nicorandil are localized in the mitochondria, although further evaluation is needed to clarify whether the grains are nicorandil or SG-86 or both.

Nicorandil has been widely used as an orally efficacious anti-anginal drug (Sakai 1989; Kinoshita & Sakai 1990; Krumenacker & Roland 1992) with the dual biological activity of a K_{ATP}-channel opener and a nitrate (Taira 1987). Garlid et al (1996) recently reported that mitochondrial K_{ATP} channels might be an important intracellular receptor for K_{ATP}-channel openers, and that they are the site of action of cardioprotective K_{ATP}-channel openers, because mitochondrial K_{ATP} channels are thought to be involved in control of mitochondrial volume and mitochondrial energetics. Many in-vivo and in-vitro experiments have demonstrated that nicorandil has cardioprotective activity when used as a K_{ATP}-channel opener (Gross 1993).

Table 1. Time course of the concentrations of nicorandil and its metabolites in the heart.

	0·25 h	0·5 h	1·0 h
Nicorandil	840·4 ± 83·2	726·2 ± 54·4	554·1 ± 35·0
SG-86	1069·1 ± 31·5	1050·0 ± 56·4**	1132·7 ± 49·4***
Unknown substances	167·1 ± 42·5***	380·0 ± 74·7*	379·5 ± 128·2

Values (ng g^{-1}) are means ± s.e.m. of four observations. [^{14}C]Nicorandil (3 mg kg^{-1}) was given orally to rats, and the metabolic profile was observed after 0·25, 0·5 and 1 h. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, compared with nicorandil content.

Table 2. Subcellular localization of nicorandil and its main metabolite, SG-86, in the heart.

	Nicorandil	SG-86
20% Homogenate	5·7 ± 0·9	6·5 ± 0·5
Mitochondria	8·2 ± 0·7	6·4 ± 0·3
Cytosol	29·3 ± 2·4	29·4 ± 5·6
Microsome	4·5 ± 0·4	4·3 ± 0·7

[^{14}C]Nicorandil (3 mg kg^{-1}) was given orally to rats, and 15 min later the rats were killed. The hearts were excised and the subcellular fractions were prepared. Values (ng (mg protein)$^{-1}$) are means ± s.e.m. of results from five rats.

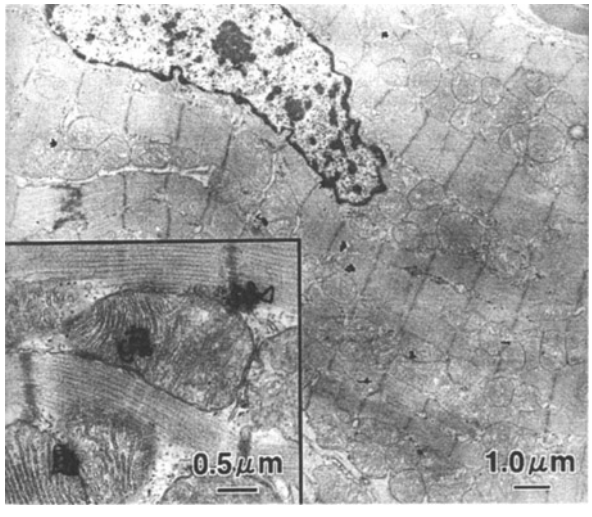

Figure 1. Appearance of ^{3}H-labelled substances in the mitochondria of the heart as revealed by electron-microscopic autoradiography. [^{3}H]Nicorandil (3 mg kg^{-1}) was given orally to rats, and 15 min later the rats were killed. Exposure for 1 month was performed at 4°C in light-tight boxes containing silica gel.

The current experiments clearly demonstrated that nicorandil given orally to rats is localized in the mitochondria, which might be the site of its cardio-protective action. Even though further investigation is required to show how the opening of mitochondrial K_{ATP} channels can exert protective effects, it is assumed that nicorandil plays an important role in cardioprotection by interacting with mitochondrial K_{ATP} channels.

References

Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., Sun, Z., Schindler, P. A. (1996) The mitochondrial K_{ATP} channel as a receptor for potassium-channel openers. J. Biol. Chem. 271: 8796–8799

Gross, J. G. (1993) The cardioprotective effects of nicorandil. Rev. Contemp. Pharmacother. 4: 199–206

Hess, B., Brand, K. (1974) In: Bergmeyer, H. V. (ed.) Methods of Enzymatic Analysis, Academic Press, New York, pp 399–409

Inoue, I., Nagase, H., Kishi, K., Higuti, T. (1991) ATP-sensitive K^+ channel in the mitochondrial inner membrane. Nature 352: 244–247

Kingsley, R. E., Cole, N. L. (1988) Preparation of cultured mammalian cells for transmission and scanning electron microscopy using Aclar film. J. Electron Microsc. Tech. 10: 77–85

Kinoshita, M., Sakai, K. (1990) Pharmacology and therapeutic effects of nicorandil. Cardiovasc. Drugs Ther. 4: 1075–1088

Krumenacker, M., Roland, E. (1992) Clinical profile of nicorandil: an overview of its hemodynamic properties and therapeutic efficacy. J. Cardiovasc. Pharmacol. 20: S93–S102

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G., Garlid, K. D. (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^+ channel from rat liver and beef heart mitochondria. J. Biol. Chem. 267: 26062–26069

Sakai, K. (1989) Nicorandil: animal pharmacology. Am. J. Cardiol. 63: J2–J10

Taira, N. (1987) Similarity and dissimilarity in the mode and mechanism of action between nicorandil and classical nitrates: an overview. J. Cardiovasc. Pharmacol. 10: S1–S9