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Inhibition of Poly (ADP-ribose) Polymerase as a Protective Effect of Nicaraven in Ionizing Radiation- and Ara-C-induced Cell Death

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Abstract. *Background: Nicaraven is a drug used for patients with a subarachnoid hemorrhage. It crosses the blood-brain barrier and has potent antivasospastic and brain-protective effects. While nicaraven scavenges the hydroxyl radical, the mechanism of its protection remains obscure. In addition to the hydroxyl radical scavenging effect, nicaraven also exhibits inhibitory action on poly (ADP-ribose) polymerase (PARP). The mechanism of the pharmacological action of nicaraven has not yet been clarified. Materials and Methods: Human myeloid HL-525 cells were exposed to ionizing radiation or hydrogen peroxide and the effect of nicaraven on the activation of the Egr-1 promoter was measured. Next, the action of the drug on DNA fragmentation and inhibition of thymidine uptake caused by the genotoxic stimulation of ionizing radiation or cytosine B-D-arabinofuranoside (ara-C) were assessed. Finally, direct inhibition of the PARP enzyme by nicaraven was measured. Results: Nicaraven did not inhibit the activation of the Egr-1 promoter caused by H_2O_2 and the activation caused by ionizing radiation. However, the drug repressed DNA fragmentation and increased thymidine uptake dose-dependently. Nicaraven had a direct inhibitory effect on PARP. Discussion: The effect of nicaraven on the Egr-1 promoter was different from that of another free-radical scavenger, N-acetyl cysteine. Nicaraven demonstrated similar protection of the PARP inhibitors including 3-aminobenzamide. Since nicaraven directly inhibits the PARP enzyme, the drug might be useful in oncology as well as in studying tissue-damaging conditions characterized by increased PARP activity.*

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Nicaraven ([+]-N,N'-propylenedinicotinamide; AVS) is a potent drug used in the treatment of patients with a subarachnoid hemorrhage (1). It has anti-vasospasmodic and brain-protective effects (2) and ameliorates ischemic cerebral edema after occlusion of the middle and internal cerebral arteries (3). The drug can penetrate the blood-brain barrier, scavenge hydroxyl radicals under physiological conditions, (4) and is used for clinical studies in Japan. Nicaraven also has a protective effect against ischemia-reperfusion injuries of various organs (5-9). Since scavenging of hydroxyl radicals is the most prominent action of the drug, the mechanism of these protective effects has been attributed mainly to this action. One characteristic feature of nicaraven is its effectiveness in protecting organs even when administered after an injury. This feature of the drug suggests an involvement of other protective mechanisms. In this context, we previously used hippocampal slice cultures and found that nicaraven protected the neuronal cells from the toxicity of excitatory amino acids (EAA) (10). In that study, we compared the effect of nicaraven on damage caused by oxygen-glucose deprivation due to N-methyl-D-aspartic acid exposure and demonstrated the possible involvement of poly (ADP-ribose) polymerase (PARP) inhibition in cell protection. PARP is directly associated with cell death events, hence it is extremely important to determine if the drug can inhibit PARP activity. We attempted to examine this action in the current study.

Materials and Methods

Cells. Human stomach adenocarcinoma cells, STKM (provided by Shunsuke Yanoma, Kanagawa Cancer Center, Japan), were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). Human HL525 myeloid leukemia cells were cultivated in RPMI-1640 medium with 20% FBS (11).

Treatment. Cells were exposed to ionizing radiation (MBR-1520R, Hitachi, Ibaraki, Japan), hydrogen peroxide, or cytosine β-D-arabinofuranoside (ara-C) with or without N-acetyl-L-cysteine (NAC), 3-aminobenzamide (3AB), theophylline, and nicaraven (provided by Chugai Pharmaceutical Co. Ltd. Tokyo, Japan). All the chemicals were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Colonogenic assay. STKM cells were treated with 0 or 5 mM of nicaraven and then exposed to 0, 5, 10, or 20 Gy of ionizing radiation. A 100 cells/ml suspension was inoculated onto 60-mm culture dishes. Fourteen days later, the colonies were fixed with methanol and stained with Giemsa-solution. The number of colonies that formed on each of the culture dishes was determined.

Adenoviral infection and reporter assay. The recombinant adenovirus, Ade.Egr-1/lacZ (12) encodes the *E. coli* lacZ gene driven by the early growth response 1 (Egr-1) gene promoter. HL525 cells were infected with the virus at the multiplicity of infection (MOI) of 10 in medium with 0.5% of FBS. Forty-two hours later, cells were treated with 30 mM of NAC, or 0.5 or 5 mM of nicaraven, and then exposed to 2 Gy of ionizing radiation or 150 μM of hydrogen peroxide. After further incubation for 6 hours, the cells were harvested and subjected to a β-galactosidase assay. In the β-galactosidase assay, 100 μg of cell lysate were mixed with 0.88 mg/ml of O-nitrophenyl-β-D-galactopyranoside, 1 mM $MgCl_2$, and 0.07 M Na_2HPO_4 (pH7.3) for 30 min at 37˚C and the reaction was stopped by the addition of 500 μl of 1 M Na_2CO_3. Absorbance of the samples were measured at 420 nm and the induced β-galactosidase activity was determined.

DNA fragmentation and tritium-thymidine uptake assay. For the DNA fragmentation analysis, 10^5 cells were lysed at 50˚C for 3 h in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.5 μg of proteinase K (Merck, Darmstadt, Germany). The digested samples were embedded in 1% low-melting agarose and applied to a 2% agarose gel and electrophoresed at 60 V in Tris-acetate-EDTA (TAE) buffer. After electrophoresis, the gels were treated with 1 μg/ml RNase solution and stained with 0.5 μg/ml ethidium bromide (13).

A tritium-thymidine uptake assay was performed on a 96-well plate containing $1x10^4$ cells per well. Ten μl of thymidine-[methyl-^{3}H](0.37 kBq/10 μl, ICN, Biochemicals, Inc. Irvine, CA, USA) was added to each well and the plates were then incubated with thymidine-[methyl-^{3}H] for 6 h. After labeling the DNA, the reactions were stopped by the addition of 200 μl of 50%-TCA and the mixtures were trapped on a GF/C glass filter (Whatman, Maidstone, UK). The trapped insoluble precipitates were washed then counted in the b spectrum in a scintillation counter (Beckman, Fullerton, CA, USA).

PARP enzyme activity inhibition assay. The effect of nicaraven on PARP was measured using the direct PARP enzyme activity inhibition assay (Trevigen, Gaithersburg, MD, USA). In brief, nicaraven, theophylline, and 3-AB were diluted with 50 mM Tris-HCl/25 mM $MgCl_2$ (pH 8.0). A 15-μl aliquot of each dilutant was mixed with the PARP enzyme and biotinylated nicotinamide adenine dinucleotide (NAD), then transferred to a 96-well plate coated with NAD-substrate on histone proteins. The plates were incubated for 30 min for ribosylation. After washing, streptavidin-conjugated horseradish peroxidase and its substrate were added and the plates were incubated for 10 min in the dark. The developed coloration was monitored at 630 nm by a plate-reader (Bio-Rad, Richmond, CA, USA).

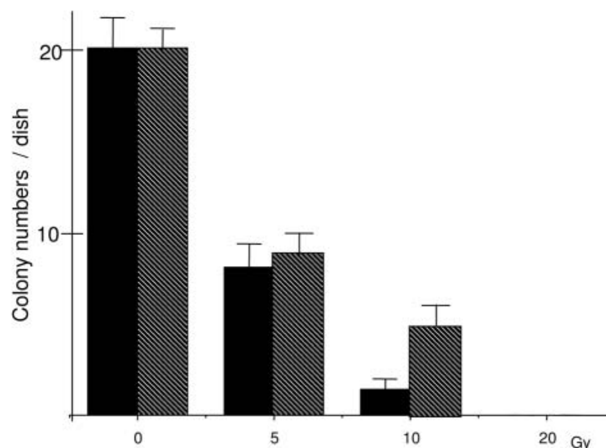

Figure 1. *Effect of nicaraven on ionizing radiation-induced toxicity in colonogenicity. Human stomach STKM cells were treated with 0 (closed column) or 5 (striped column) mM of nicaraven and exposed to different doses of ionizing radiation. The numbers of formed colonies were determined. The data are expressed as the mean of 3 experiments; bars, S.D.*

Results

Initially, we attempted to determine whether nicaraven could ameliorate the toxic effect of radicals induced by ionizing radiation on self-renewing colonogenic ability (Figure 1). Nicaraven alone did not alter the colonogenic capability of the cells. When the cells were exposed to ionizing radiation, the number of colonies that formed decreased directly with the dose of ionizing radiation. Nicaraven reduced the toxic effect of 5 and 10 Gy ionizing radiation. While the effect was not significant at 5 Gy, nicaraven significantly mitigated the self-renewing capability at 10 Gy ($p<0.002$). A higher dose of ionizing radiation yielded no colonies at all, even with nicaraven.

Since nicaraven protected cells from moderate doses of ionizing radiation, we next addressed whether nicaraven could suppress the activation of Egr-1 promoter induced by ionizing radiation or hydrogen peroxide. Cells were infected with Ade.Egr-1/lacZ, treated with nicaraven or NAC, exposed to ionizing radiation or hydrogen peroxide, and then the marker protein expression was compared (Figure 2). Treatment of cells with NAC, or nicaraven, alone did not activate the promoter. Both ionizing radiation and hydrogen peroxide activated the promoter as previously reported (14). The free-radical scavenger, NAC, inhibited this activation in both cases. However, unlike NAC, nicaraven did not modulate promoter activation caused by ionizing radiation or hydrogen peroxide.

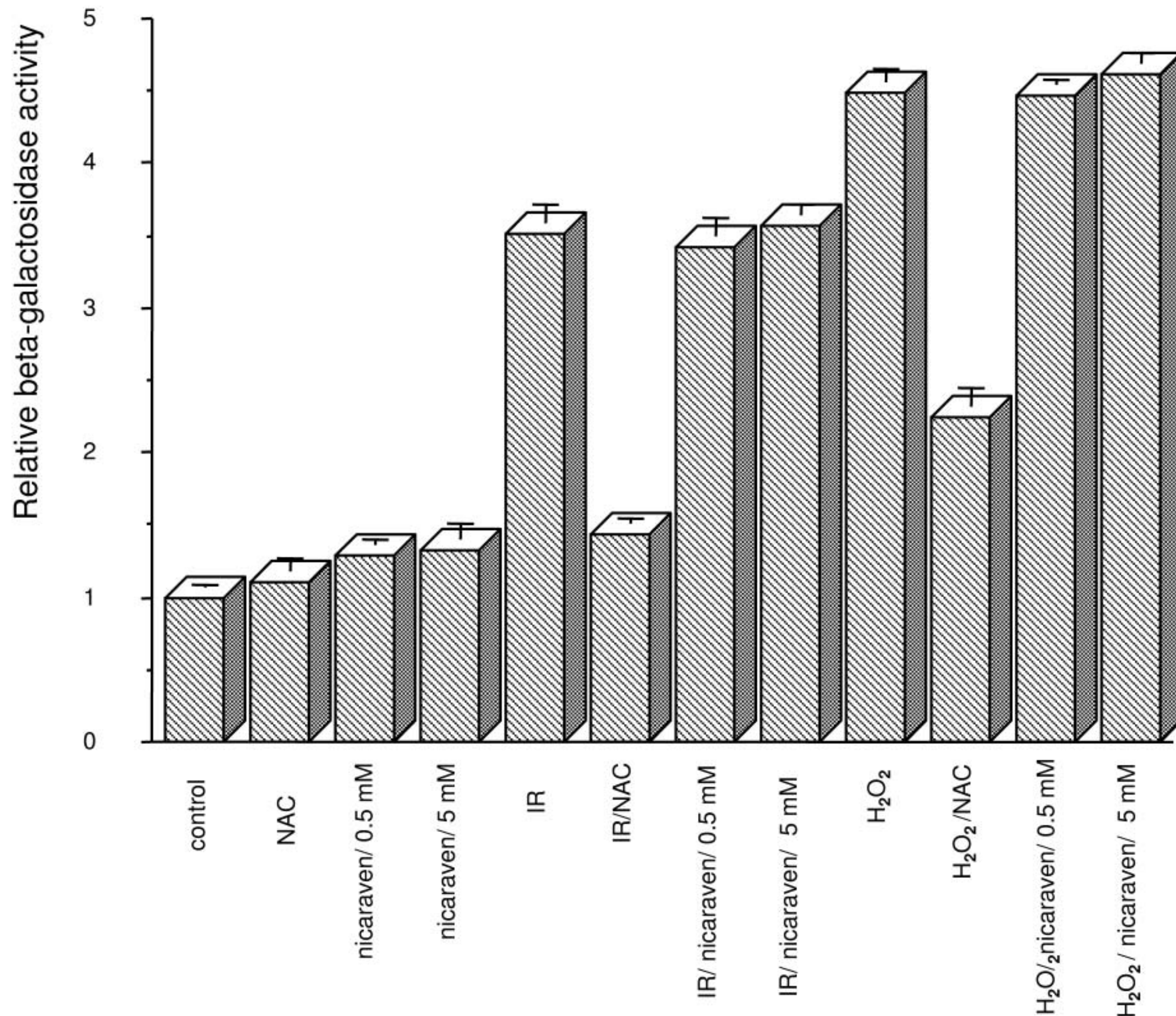

Figure 2. *Effect of nicaraven on Egr-1 promoter activated by ionizing radiation or hydrogen peroxide. HL525 cells were infected with Ade.Egr-1/lacZ at MOI10, treated with nicaraven or NAC, and exposed to ionizing radiation (2 Gy) or hydrogen peroxide (150 min). Expressed β-galactosidase activities were assayed and Egr-1 promoter activation was determined. The data are expressed as the mean of 3 experiments; bars, S.D.*

Ionizing radiation induces DNA fragmentation and inhibits DNA synthesis in the cells. The effects of nicaraven on DNA fragmentation and inhibition of DNA synthesis were measured. Cells were treated with nicaraven and exposed to 2 Gy of ionizing radiation. The PARP inhibitor, 3-AB, was used as a control (Figure 3A). Ionizing radiation induced DNA fragmentation. Nicaraven inhibited this fragmentation in a dose-dependent manner. Like 3-AB, nicaraven was also found to have blocked the inhibition of DNA synthesis when assayed using tritium-thymidine uptake (Figure 3B). Notably, nicaraven treatment had a protective effect even when applied after the irradiation (Figure 3A). This result was consistent with that of a previous report (15).

Since nicaraven was proven to inhibit the DNA fragmentation or recover the DNA synthesis caused by ionizing radiation, this effect was also examined on another genotoxic agent, ara-C. Ara-C is an antimetabolite and, when incorporated, causes chain termination in DNA synthesis. Ara-C induced DNA fragmentation in STKM cells (Figure 4A). Like 3-AB, this fragmentation was also inhibited by nicaraven. When DNA synthesis was evaluated, nicaraven blocked the inhibition of tritium-thymidine uptake induced by ara-C treatment (Figure 4B). These results were comparable to data obtained after exposure to ionizing radiation. The extent of the protective effect of nicaraven was similar to that of 3-AB.

Since nicaraven protected the cells from two different types of genotoxic stimulation, we measured the effect of nicaraven on PARP-inhibition by the direct inhibition assay to determine if nicaraven can inhibit PARP. The result revealed that

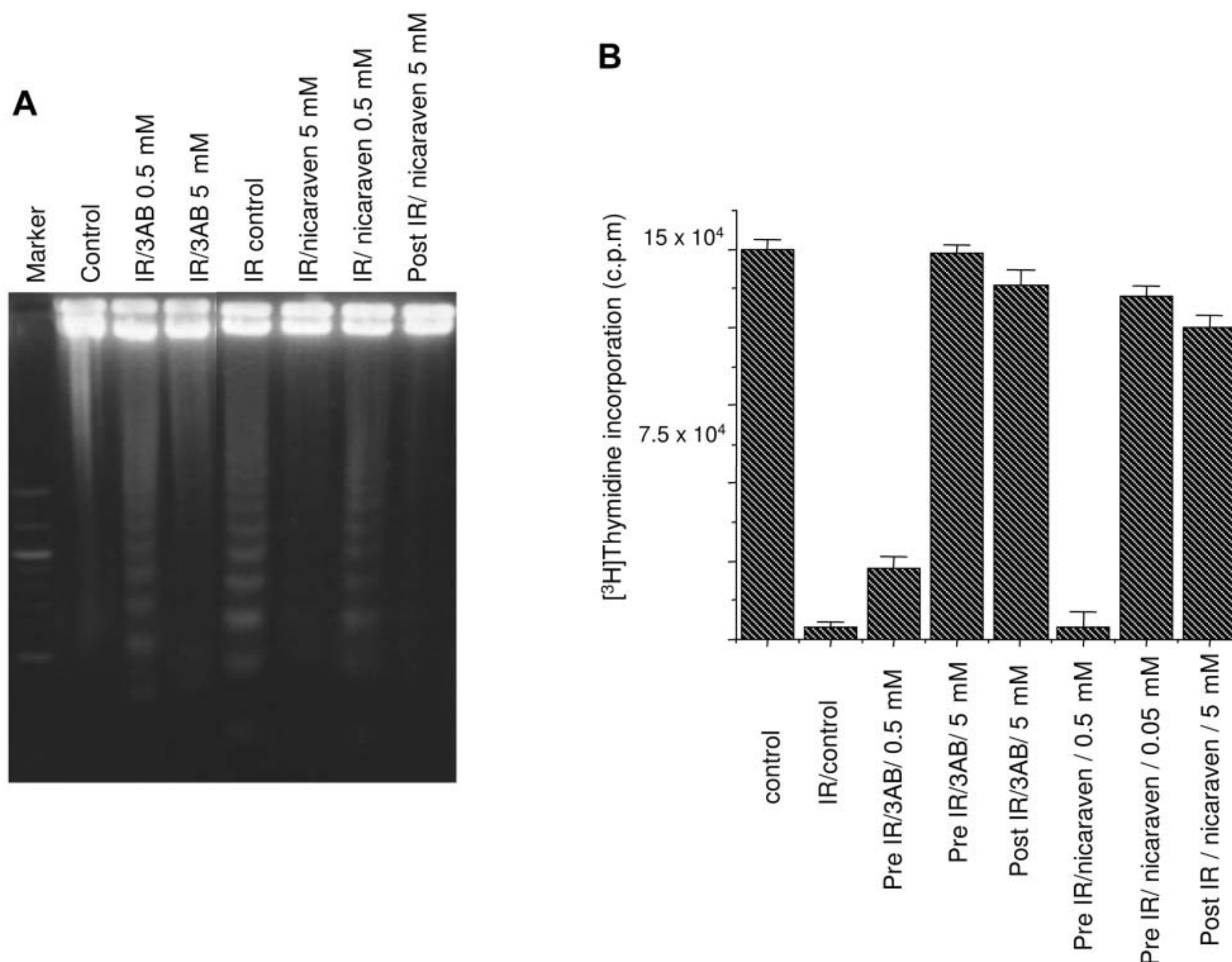

Figure 3. *Effect of nicaraven on DNA fragmentation (A) and thymidine uptake (B): A, with ionizing radiation, B, with ara-C. #The data are expressed as the mean of 3 experiments; bars, S.D.*

nicaraven inhibited PARP enzyme activities (Figure 5). When the effect of nicaraven was compared to that of other PARP-inhibitors, 3-AB had the most potent effect on inhibition. Nicaraven was less active than theophylline. However, nicaraven inhibited PARP at a working concentration. The 50% inhibitory concentration (IC50) was approximately 1.5 mM.

Discussion

In the present study, we demonstrated that nicaraven can inhibit PARP via a mechanism other than by scavenging radicals. Our first result demonstrated that nicaraven protected cells from the noxious stimulation of ionizing radiation. It improved colonogenicity, and subsequent results demonstrated the inhibition of DNA fragmentation and the recovery of thymidine uptake.

The protection observed, in the past has been considered a consequence of the well-known free-radical scavenging effect of nicaraven. When genotoxic ionizing radiation causes cell damage, the primary response of the irradiated cells is activation of the early responsive genes, such as Egr-1, which encodes the transcription factor for further activation of the downstream repair enzymes. This activation is mediated by reactive oxygen intermediates via the $CC(A/T)_6GG$ (CArG) elements (11, 14). However, our data indicated that nicaraven could not modulate the activation of the Egr-1 promoter resulting from ionizing radiation or hydrogen peroxide. The result of nicaraven contrasted with the data of NAC, which is known to capture radicals in its own cysteine residue. The activation was not inhibited by nicaraven even when the concentration was increased to 5 mM. This was the same concentration as that causing

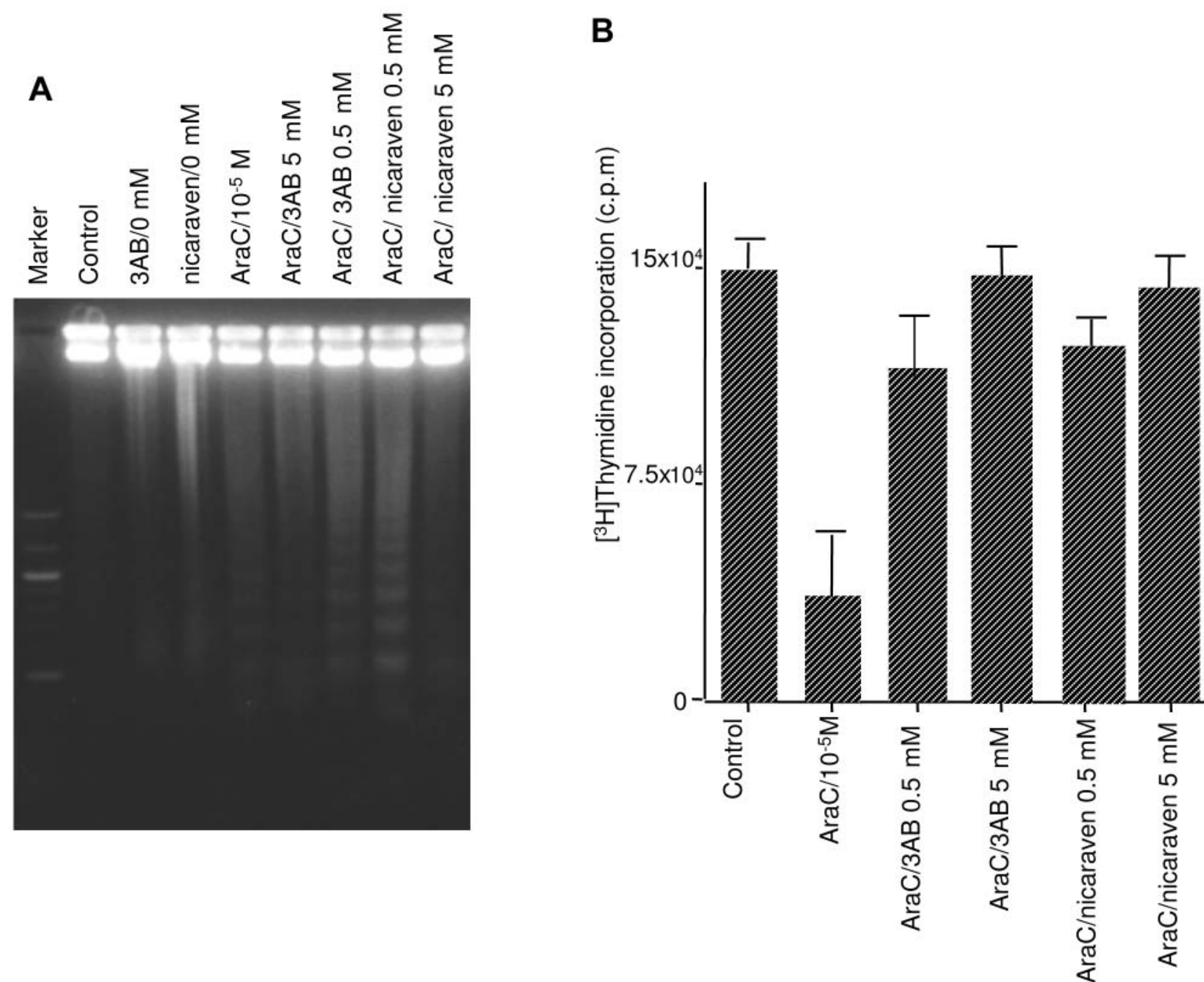

Figure 4. *Effect of nicaraven on DNA fragmentation (A) and thymidine uptake (B) with ara-c. #The data are expressed as the mean of 3 experiments; bars, S.D.*

colonogenicity, DNA fragmentation, or thymidine uptake. Our results implied that nicaraven did not interfere with the pathway of Egr-1 activation through the CArG elements.

Exposure of the cells to both ionizing radiation and hydrogen peroxide induced activation of the early response genes. Twenty Gy of ionizing radiation or 150 μM of hydrogen peroxide up-regulated transcription of early response genes (16). However, in a cell death pathway, each stimulation has a quite different effect on the host. While ionizing radiation induces fragmentation of DNA in the cells, hydrogen peroxide does not cause fragmentation (13). Since nicaraven did not inhibit activation of the promoter of the early response gene, we examined the effect of nicaraven on DNA fragmentation. In addition, tritium thymidine uptake into DNA was examined since ionizing radiation also inhibits DNA synthesis.

The results demonstrated that nicaraven inhibited DNA fragmentation and restored thymidine uptake which had been reduced by ionizing radiation. Moreover, nicaraven protected the cells from ara-C induced genotoxicity. The cytotoxicity of ara-C is unlikely to be inhibited by most free-radical scavengers although radicals are generated when cells are dying.

Previously, we discovered the effect of nicaraven against N-methyl-D-aspartic acid (NMDA)-induced hippocampal cell death in organotypic slice cultures (10). Neuronal cell death induced by oxygen-glucose depravation was inhibited by nicaraven and NAC. However, we found that excitotoxic

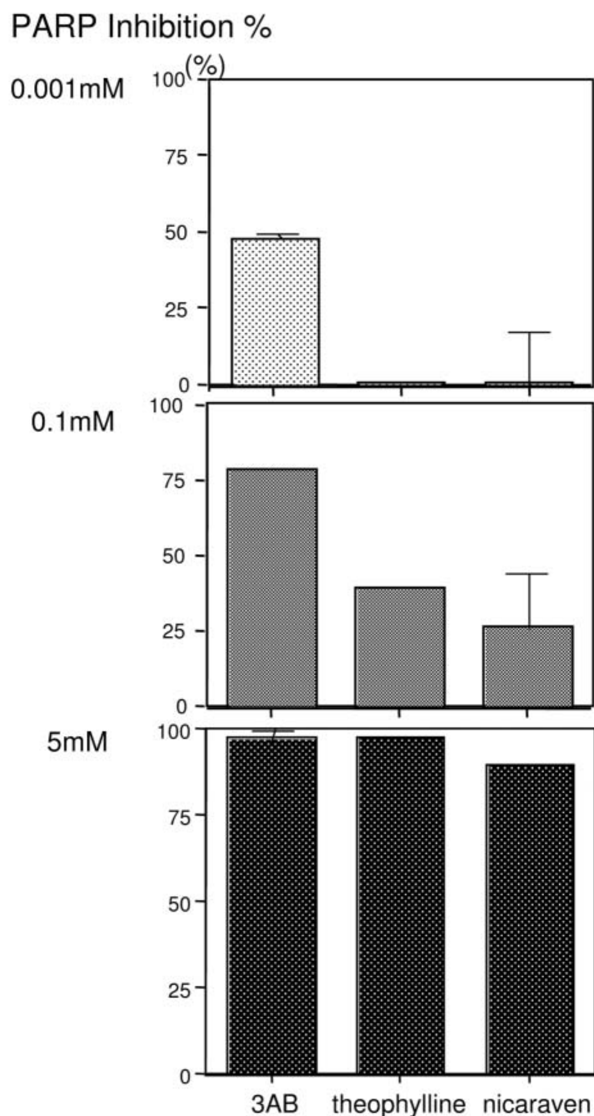

Figure 5. *Direct PARP inhibition assay. PARP enzyme activity was measured by incorporation of a unique biotinated NAD substrate into histone proteins. A comparison was made of PARP inhibition by nicaraven and two known PARP inhibitors, 3-AB and theophylline.*

pyramidal cell death by NMDA was protected only by nicaraven. In the process, we noticed the similarity between the effects of nicaraven and 3-AB or theophylline and discussed the possible role of nicaraven as a PARP inhibitor. The results of ara-C further supported this notion.

Our final result provided the information that nicaraven is a direct inhibitor of PARP action on histone proteins. PARP, also called poly (ADP-ribose) synthetase (PARS), is the enzyme catalyzing the NAD-dependent addition of ribose to adjacent nuclear proteins. It also plays an important role in DNA repair, but can also lead the cell into death by depleting the cellular NAD pool. Under physiological conditions, PARP maintains genomic stability and is one of the substances for caspase-3 action in apoptosis. Under pathological states, PARP mediates cell death after ischemia-reperfusion injury, glutamate excitotoxicity, and various inflammatory processes (17-19). It was found to be up-regulated by transient cerebral ischemia (20), and early ischemia/reperfusion (IR) injury in heart transplantation (21). PARP-inhibition protected cells from these injuries (22) much like the results of our study.

The concentration at which PARP inhibition occurred is relevant to the concentration obtained in a clinical study. When patients were treated with continuous infusion of 6 g of nicaraven for 6 h (1 g/h), the peak drug concentration almost reached 60 μg/ml at two hours after infusion. In this condition, the blood concentration of the drug exceeded 35 μg/ml in the period from 1 to 8 h after infusion (unpublished data, provided by Chugai Pharmaceutical Co. Ltd. Tokyo, Japan). The concentrations corresponded to 0.21 (25% inhibition of the PARP in our result) and 0.12 mM (20% inhibition in our result), respectively. So far, in the literature the effect of nicaraven on PARP has not been discussed, except for a report describing the effect as slight inhibition (23). Further investigation of the effect may clarify the mechanism of its potent cellular protection and since nicaraven was proven to have a direct PARP inhibitory effect, the drug might be useful in oncology, as well as in studying tissue-damaging conditions characterized by PARP activation.

References

1 Asano T, Takakura K, Sano K, Kikuchi H, Nagai H, Saito I, Tamura A, Ochiai C and Sasaki T: Effects of a hydroxyl radical scavenger on delayed ischemic neurological deficits following aneurysmal subarachnoid hemorrhage: results of a multicenter, placebo-controlled double-blind trial. J Neurosurg *84*: 792-803, 1996.

2 Germano A, Imperatore C, d'Avella D, Costa G and Tomasello F: Antivasospastic and brain-protective effects of a hydroxyl radical scavenger (AVS) after experimental subarachnoid hemorrhage. J Neurosurg *88*: 1075-1081, 1998.

3 Asano T, Johshita H, Koide T and Takakura K: Amelioration of ischaemic cerebral oedema by a free-radical scavenger, AVS: 1,2-bis(nicotinamido)-propane. An experimental study using a regional ischaemia model in cats. Neurol Res *6*: 163-168, 1984.

4 Akimoto T: Quantitative analysis of the kinetic constant of the reaction of N,N'-propylenedinicotinamide with the hydroxyl radical using dimethyl sulfoxide and deduction of its structure in chloroform. Chem Pharm Bull (Tokyo) *48*: 467-476, 2000.

5 Alam MS, Ku K, Yamauchi M, Hashimoto M, Nosaka S, Hossain MS, Masumura S, Nakayama K and Tamura K: Protective effects of nicaraven, a new hydroxyl radical scavenger, on the endothelial dysfunction after exposure of pig coronary artery to hydroxyl radicals. Mol Cell Biochem *178*: 237-243, 1998.

6 Alam MS, Ku K, Hashimoto M, Nosaka S, Saitoh Y, Yamauchi M, Masumura S, Nakayama K and Tamura K: Hydroxyl radical scavenging effect of nicaraven in myocardial and coronary endothelial preservation and reperfusion injury. Cardiovasc Res *33*: 686-692, 1997.
7 Ku K, Kin S, Hashimoto M, Saitoh Y, Nosaka S, Iwasaki S, Alam MS and Nakayama K: The role of a hydroxyl radical scavenger (nicaraven) in recovery of cardiac function following preservation and reperfusion. Transplantation *62*: 1090-1095, 1996.
8 Masaki Y, Kumano K, Endo T, Iwamura M, Koshiba K, Yokota K and Okubo M: Protective effect of nicaraven against prolonged cold kidney preservation and reperfusion injury. Transplant Proc *30*: 3758-3760, 1998.
9 Yokota R, Fukai M, Shimamura T, Suzuki T, Watanabe Y, Nagashima K, Kishida A, Furukawa H, Hayashi T and Todo S: A novel hydroxyl radical scavenger, nicaraven, protects the liver from warm ischemia and reperfusion injury. Surgery *127*: 661-669, 2000.
10 Yoshinaga H, Watanabe M and Manome Y: Possible role of nicaraven in neuroprotective effect on hippocampal slice culture. Can J Physiol Pharmacol *81*: 683-689, 2003.
11 Datta R, Taneja N, Sukhatme VP, Qureshi SA, Weichselbaum R and Kufe DW: Reactive oxygen intermediates target CC(A/T)6GG sequences to mediate activation of the early growth response 1 transcription factor gene by ionizing radiation. Proc Natl Acad Sci USA *90*: 2419-2422, 1993.
12 Manome Y, Kunieda T, Wen PY, Koga T, Kufe DW and Ohno T: Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the Egr-1 promoter: activation by ionizing radiation or uptake of radioactive iododeoxyuridine. Hum Gene Ther *9*: 1409-1417, 1998.
13 Manome Y, Datta R, Taneja N, Shafman T, Bump E, Hass R, Weichselbaum R and Kufe D: Coinduction of c-jun gene expression and internucleosomal DNA fragmentation by ionizing radiation. Biochemistry *32*: 10607-10613, 1993.
14 Datta R, Rubin E, Sukhatme V, Qureshi S, Hallahan D, Weichselbaum RR and Kufe DW: Ionizing radiation activates transcription of the EGR1 gene via CArG elements. Proc Natl Acad Sci USA *89*: 10149-10153, 1992.
15 Imperatore C, Germano A, d'Avella D, Tomasello F and Costa G: Effects of the radical scavenger AVS on behavioral and BBB changes after experimental subarachnoid hemorrhage. Life Sci *66*: 779-790, 2000.
16 Manome Y, Datta R and Fine HA: Early response gene induction following DNA damage in astrocytoma cell lines. Biochem Pharmacol *45*: 1677-1684, 1993.
17 Gilliams-Francis KL, Quaye AA and Naegele JR: PARP cleavage, DNA fragmentation, and pyknosis during excitotoxin-induced neuronal death. Exp Neurol *184*: 359-372, 2003.
18 Lam TT, Abler AS, Kwong JM and Tso MO: N-methyl-D-aspartate (NMDA)-induced apoptosis in rat retina. Invest Ophthalmol Vis Sci *40*: 2391-2397, 1999.
19 Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM and Dawson VL: Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science *297*: 259-263, 2002.
20 Narasimhan P, Fujimura M, Noshita N and Chan PH: Role of superoxide in poly(ADP-ribose) polymerase upregulation after transient cerebral ischemia. Brain Res Mol Brain Res *113*: 28-36, 2003.
21 Fiorillo C, Ponziani V, Giannini L, Cecchi C, Celli A, Nediani C, Perna AM, Liguori P, Nassi N, Formigli L, Tani A and Nassi P: Beneficial effects of poly (ADP-ribose) polymerase inhibition against the reperfusion injury in heart transplantation. Free Radic Res *37*: 331-339, 2003.
22 Lo EH, Bosque-Hamilton P and Meng W: Inhibition of poly(ADP-ribose) polymerase: reduction of ischemic injury and attenuation of N-methyl-D-aspartate-induced neurotransmitter dysregulation. Stroke *29*: 830-836, 1998.
23 Zingarelli B, Scott GS, Hake P, Salzman AL and Szabo C: Effects of nicaraven on nitric oxide-related pathways and in shock and inflammation. Shock *13*: 126-134, 2000.

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