Baicalin Promoted the Repair of DNA Single Strand Breakage Caused by H₂O₂ in Cultured NIH3T3 Fibroblasts

Xin Chen, Hiroshi Nishida, and Tetsuya Konishi

Department of Radiochemistry-Biophysics and Functional Food Sciences, Niigata University of Pharmacy and Applied Life Sciences (NUP ALS); 5–13–2 Kamishin-ei, Niigata 950–2081, Japan.

To whom correspondence should be addressed. e-mail: konishi@niigata-pharm.ac.jp © 2003 Pharmaceutical Society of Japan

Received July 19, 2002; accepted October 21, 2002

DNA lesions have been implicated in many pathological processes, including mutagenesis, carcinogenesis and aging. Normal cellular metabolism results in the continuous generation of reactive oxygen species (ROS), such as superoxide radical or nonradical hydrogen peroxide. The hydroxyl radical is the most reactive toward biological molecules and produces multiple modifications in DNA molecules, such as base and sugar damage, and DNA protein cross-link. The DNA damage is continuously ongoing, but can also be efficiently repaired in the cells.

Recently, much attention has been paid to the roles of small antioxidant molecules of natural origin, such as flavonoids in the protection of oxidative DNA damage in addition to their wide variety of biological functions. Baicalin, a flavonoid isolated from the root of Scutellaria baicalensis G., has also been demonstrated to have multiple biological functions, such as anti-inflammatory activity, inhibition of aldose reductase, inhibition of HIV infection, and nitric oxide producing activity. Baicalin also has a strong antioxidant activity toward ROS, including hydroxyl radical (OH), superoxide anions (O₂⁻), and peroxynitrite (ONOO⁻), and inhibits lipid peroxidation in rat tissues. However, few studies have been done on the effect of Baicalin in oxidative DNA lesions in the cell. The degree of DNA damage, as assessed by the Comet assay, is considered to represent the capacity of the cells to resist H₂O₂ stress or to repair the single strand break (SSB) formed.

In the present study, we examined the formation and subsequent repair of SSB in the NIH3T3 fibroblast cell line stressed by H₂O₂ and the effect of Baicalin on these processes by the Comet assay.

MATERIALS AND METHODS

Chemicals Growth medium, Dulbecco’s Modified Eagle’s Medium (DMEM) and Fetal Bovine Serum (FBS) were pur-

chased from Sigma Chemical Co., Ltd. Antibiotics and antifungotics were from Gibco BRL Co., Ltd. Cell culture dishes were obtained from Corning Co. Ltd. Comet Assay kit was from Trevigen, Co. Ltd. SYBR green and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) were from Dojindo Co., Ltd. Purified Baicalin was generously provided from the Beijing Institute of Pharmacy, Academia Sinica. All other chemicals used were of analytical grade.

Cell Culture and Treatment NIH3T3 fibroblasts (Riken Cell Bank, Japan) were grown in DMEM with 10% FBS at 37 °C, under a 95% air and 5% CO₂ atmosphere in culture dishes (1—2×10⁵ cells in Ø35 mm). The cells (1—2×10⁵ cells) were incubated in fresh DMEM containing 10% FBS at 37 °C for 24 h in the absence and presence of Baicalin. Then, the medium was replaced for fresh DMEM without FBS, and the cells were treated with defined concentrations of H₂O₂ for 15 min. The medium was replaced again with fresh medium containing 10% FBS and then incubated for the various time periods indicated. Finally, the cells were collected and subjected to Comet assay.

Single Cell Gel Electrophoresis and Scoring (Comet Assay) Comet assay was carried out using the Comet Assay kit provided by Trevigen, Inc., and according to Trevigen instruction protocol. Briefly, the cells (1×10⁵ cells/ml) suspended in ice-cold PBS were mixed with molten low melting agarose and placed on a cometslide glass. The agar incorporated cells were treated with pre-chilled lysis buffer for 60 min on ice and then immersed in freshly prepared alcali solution for 60 min in the dark at room temperature. The electrophoresis was performed at 1 V/cm for 10 min in the TBE buffer (1% Tris Base, 0.5% Boric Acid, 0.093% EDTA). Cellular DNA was stained by SYBR Green and visualized under an epifluorescence microscope (OLYMPUS model BH2-RFCA). The damage profile of DNA was assessed by visual scoring of the tail length of the comets ran-
domly observed for at least 100 cells on each slide.

The scoring method for the damaged DNA followed the method reported by Mihalis *et al.* The DNA damage images were classified into 5 grades (from class 0 to 4) shown in Fig. 1, and each comet was assigned to one of the damage grades according to the visualized shape apparent in the damage histogram. Overall DNA damage was evaluated by the combined fractional % of class 2 to 4 damage.

**Cell Viability Measurement by MTT** Cell viability was detected by the MTT method as described previously. Cells (3–6×10⁴) grown in DMEM containing 10% FBS for 20 h in 48-well plastic culture plates were incubated for another 24 h in the presence or absence of Baicalin. After washing the cells with fresh DMEM without FBS twice, the cells were treated with 0.5 mM H₂O₂ in the above FBS-free medium for the time periods indicated. After replacing the medium for fresh DMEM containing 10% FBS, the cell viability was quantified by MTT. The cell viability was given as the % of sham-treated normal cell cultures and the mean ± S.D. of 6 different cultures.

**Statistical Analysis** The General Linear Model procedure of SAS (1982) combined with Duncan’s multiple range test comprised the statistical analysis. The level of statistical significance was taken as *p* < 0.05.

**RESULT AND DISCUSSION**

NIH3T3 fibroblast grown in DMEM containing 10% FBS in a plastic culture dish was washed with fresh FBS-free DMEM and it was then pulse-chased for 15 min with 0.5 mM H₂O₂ in FBS-free DMEM at 37 °C. The cells were washed and replaced with H₂O₂-free DMEM containing 10% FBS, then the DNA damage was determined by Comet assay at a defined time after successive incubations at 37 °C. The comet tail length was measured as the index of DNA SSB, and the damage grades were classified into the 5 categories as shown in Fig. 1. The damage grade histogram revealed that DNA was significantly damaged after the H₂O₂ abuse, as shown in Fig. 2A, in that the proportion of grade 3 and 4 lesions was remarkably increased with the loss of class 0 and 1 DNA. When overall DNA damage was evaluated with the fractional % of class 2 to 4 damage altogether, the DNA lesion increased with increased concentrations of H₂O₂ (0.1 to 0.5 mM range), and reached approximately 15 times the untreated control cells with 0.5 mM H₂O₂ (data not shown).

The DNA damage itself was not significantly prevented when the cells were pre-incubated with Baicalin for 24 h before 0.33 mM H₂O₂ abuse. It was also noted that cells showed a slight increase in class 1 DNA lesion after Baicalin treatment for 24 h compared to untreated cells (Fig. 2B). The time dependent changes of the damage histograms were quite different between the control and Baicalin-treated cells when the H₂O₂ treated cells were further incubated in fresh DMEM with 10% FBS (Figs. 2A, B). The SSB lesions were significantly repaired during the incubation time, even in the control cells, as was shown in the damage histogram, in that class 3 and 4 damage fractions were shifted back into a class 1 damage fraction. The recovery of the damage histogram was more marked in those cells pretreated with 100 µM Baicalin before the H₂O₂ abuse (Fig. 2B), although the complete repair of the SSB (to class 0 damage) was not attained.

---

**Fig. 1. Grade Classification of H₂O₂-Induced DNA Damage in NIH3T3 Fibroblast Cell**

Cells (2×10⁴ cells/dish) grown in DMEM containing 10% FBS in 35 mm plastic culture dishes were treated with 0.33 mM H₂O₂ in FBS-free DMEM for 15 min. Comet assay was carried out as described in the Methods section.

**Fig. 2. Effect of Baicalin on DNA Damage Caused by H₂O₂ in NIH3T3 Fibroblast**

Cells (1×10⁴/dish) were incubated for 24 h with (B) or without (A) 100 µM Baicalin. Then the cells were re-suspended in fresh FBS-free DMEM, and pulse-chased with 0.33 mM H₂O₂ for 15 min. After the medium was replaced with the fresh DMEM containing 10% FBS, the cells were incubated for the time period indicated at 37 °C and subjected to the Comet assay. Fractional % of the class 2 to 4 DNA damage histogram was re-plotted as an index of overall DNA damage (C). Data are given as mean±S.D. of three independent experiments. *p < 0.05.*
even after 120 min incubation (data not shown). The Baicalin-dependent promotion of DNA damage repair was more clearly demonstrated in Fig. 2C, in that the DNA lesion was expressed as the fractional % of class 2 to 4 damages altogether. Almost the same results were obtained in the cells pre-treated with 50 μM Baicalin, indicating the effect of Baicalin was saturated even with its 50 μM concentration (data not shown).

The cell viability after H$_2$O$_2$ abuse and the protective effect of Baicalin were examined by the MTT staining method. The cells were chased with 0.5 mM H$_2$O$_2$ for 2 h, because no significant cell death was observed by the 15 min treatment, as far as the cell death was detected by MTT method (data not shown). The cell viability decreased to approximately 77% of the control after H$_2$O$_2$ abuse; however, the viability was slightly but significantly recovered to 83 and 84%, respectively, in the cells pre-treated with 50 and 100 μM Baicalin for 24 h before the H$_2$O$_2$ abuse (Fig. 3).

The present study revealed that Baicalin enhanced the DNA damage repair in NIH3T3 fibroblast cells when they were incubated for 24 h before the H$_2$O$_2$ abuse, although it did not give rise to significant protection against H$_2$O$_2$-dependent damage production. Hydrogen peroxide is known to diffuse into the cell nucleus and cause damage in DNA by generating an OH radical in close proximity to the DNA.\textsuperscript{21} This was proved also in the present experiments, in that only 15 min exposure to 0.5 mM H$_2$O$_2$ was enough to cause significant SSB production in NIH3T3 fibroblast, and the H$_2$O$_2$ effect was concentration dependent (data not shown). Therefore, it is indicated that simple scavenging of ROS by Baicalin was not the major cause of the DNA damage protection, although the antioxidant activity of Baicalin was shown elsewhere.\textsuperscript{19} It is more likely that Baicalin improved cellular antioxidant activity against H$_2$O$_2$ stress by modifying the repair potential of damaged DNA, thus functioning as a biological response modifier. However, the mechanism of the enhanced protection against DNA damage seems rather complex. As shown in Fig. 2, Baicalin slightly induced the accumulation of class 1 damage frequency by the pre-incubation for 24 h, indicating that some DNA damage in the cells has already occurred before H$_2$O$_2$ abuse, though it is not critical. Ueda et al.\textsuperscript{20} reported the prooxidant effect of Baicalin that induced apoptosis by the marginal generation of intracellular ROS up to 24 h incubation, and they suggested that this is a cause of enhanced cellular antioxidant potential during pre-incubation with Baicalin. Therefore, further discussion is needed to conclude that Baicalin acted directly on the DNA damage repair process, or indirectly via a production of marginal ROS by its prooxidant action because DNA damage repair enzymes are known to be expressed by ROS.\textsuperscript{20} Cells have evolved a number of mechanisms to rescue them from oxidative stress. DNA damage repair is one of them. The DNA repair mechanism involves base and nucleotide excision repairs, in that several repair enzymes operate, such as DNA glycosylase, DNA helicase, and DNA polymerase and ligase.\textsuperscript{22,23} It is interesting to know that Baicalin taken up into the cell exerts its protective effects either by modifying cellular signal transduction pathways or by activating DNA repairing enzymes through manipulating the antioxidant re-

![Fig. 3. Protective Effect of Baicalin on H$_2$O$_2$ Induced Cell Death in NIH3T3 Fibroblast](image)

Cells (1×10$^6$/well) grown either with or without Baicalin for 24 h were treated with 0.5 mM H$_2$O$_2$ for 2 h. The cell bioavailability was determined by the MTT staining method. Data are the means±S.D. of 6 cell cultures. p<0.05.

**REFERENCES**

1) Present address: College of Traditional Chinese Medicine and Pharmacology, Capital University of Medicinal Science, No. 22 Hepingli Northstreet, Dongcheng, Beijing, 100013, China.


