Baicalin Promoted the Repair of DNA Single Strand Breakage Caused by H_2O_2 in Cultured NIH3T3 Fibroblasts

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The protective effect of Baicalin, a flavonoid isolated from the root of *Scutellaria baicalensis* G., on H_2O_2 -induced DNA single strand break (SSB) was examined in NIH3T3 mouse fibroblasts by Comet assay (single cell gel electrophoresis technique). When the cells were pulse-chased with H_2O_2 (0.1–0.5 mM) for 15 min in fetal bovine serum (FBS)-free Dulbecco's Modified Eagle's Medium (DMEM), SSB occurred in the DNA as reported elsewhere in dose-dependent manner. Baicalin (50, 100 μ M) which was incubated with the cells for 24 h before the H_2O_2 chase did not give rise to significant protection against the SSB formation. However, when the time required to cause a change in the DNA damage histogram obtained by the Comet assay was precisely examined after the H_2O_2 chase, it was found that the H_2O_2 induced SSB was more promptly repaired in the cells pretreated with Baicalin prior to the H_2O_2 chase, compared to untreated control cells. At the same time, the cell viability examined by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay after the H_2O_2 abuse was moderately recovered in the Baicalin increased by the Baicalin treatment. It was thus concluded that Baicalin that was known as an antioxidant flavonoid *in vitro* also functions as a biological response modifier, improving the cellular repair potential of oxidatively damaged DNA.

Key words DNA damage repair; NIH3T3 fibroblast; baicalin; comet assay; DNA SSB (single strand break)

DNA lesions have been implicated in many pathological processes, including mutagenesis, carcinogenesis and aging.²⁾ Normal cellular metabolism results in the continuous generation of reactive oxygen species (ROS), such as superoxide radical or nonradical hydrogen peroxide.³⁾ 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.⁴⁾ The DNA damage is continuously ongoing, but can also be efficiently repaired in the cells.^{5,6)}

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.⁷⁾ 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,⁸⁾ inhibition of aldose reductase,^{9,10)} inhibition of HIV infection,¹¹⁾ and nitric oxide producing activity.¹²⁾ Baicalin also has a strong antioxidant activity toward ROS, including hydroxyl radical ($OH\cdot$), superoxide anions ($O_2\cdot^-$) and peroxynitrite ($ONOO^-$),^{13–15)} and inhibits lipid peroxidation in rat tissues.¹⁶⁾ 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 H_2O_2 stress or to repair the single strand break (SSB) formed.¹⁷⁾

In the present study, we examined the formation and subsequent repair of SSB in the NIH3T3 fibroblast cell line stressed by H_2O_2 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 antimycotics 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% CO_2 atmosphere in culture dishes ($1-2 \times 10^5$ cells in $\phi 35$ mm). The cells ($1-2 \times 10^5$ 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 H_2O_2 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 (1×10^5 cells/ml) suspended in ice-cold PBS were mixed with molten low melting agarose and placed on a comet slide glass. The agar incorporated cells were treated with pre-chilled lysis buffer for 60 min on ice and then immersed in freshly prepared alkali 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-

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domly observed for at least 100 cells on each slide.

The scoring method for the damaged DNA followed the method reported by Mihalics *et al.*¹⁷⁾ 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.¹⁸⁾ Cells ($3-6 \times 10^4$) 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 H_2O_2 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 $\pm$ 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 H_2O_2 in FBS-free DMEM at 37°C. The cells were washed and replaced with H_2O_2 -free DMEM containing 10% FBS, then the DNA lesion 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 H_2O_2 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 H_2O_2 (0.1 to 0.5 mM range), and reached approximately 15 times the untreated control cells with 0.5 mM H_2O_2 (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 H_2O_2 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 H_2O_2 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 μ M Baicalin before the H_2O_2 abuse (Fig. 2B), although the complete repair of the SSB (to class 0 damage) was not attained,

Fig. 1. Grade Classification of H_2O_2 -Induced DNA Damage in NIH3T3 Fibroblast Cell

Cells (2×10^5 cells/dish) grown in DMEM containing 10% FBS in 35 mm ϕ plastic culture dishes were treated with 0.33 mM H_2O_2 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 H_2O_2 in NIH3T3 Fibroblast

Cells (1×10^5 /dish) were incubated for 24 h with (B) or without (A) 100 μ M Baicalin. Then the cells were re-suspended in fresh FBS-free DMEM, and pulse-chased with 0.33 mM H_2O_2 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 means $\pm$ 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_2O_2 abuse and the protective effect of Baicalin were examined by the MTT staining method. The cells were chased with 0.5 mM H_2O_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_2O_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_2O_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_2O_2 abuse, although it did not give rise to significant protection against H_2O_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.²⁾ This was proved also in the present experiments, in that only 15 min exposure to 0.5 mM H_2O_2 was enough to cause significant SSB production in NIH3T3 fibroblast, and the H_2O_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.¹⁹⁾ It is more likely that Baicalin improved cellular antioxidant activity against H_2O_2 stress by modulating 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_2O_2 abuse, though it is not critical. Ueda *et al.*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.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.^{22,23)} It is interesting to know that Baicalin taken up into the cell exerts its protective effects either by modulating cellular signal transduction pathways or by activating DNA repairing enzymes through manipulating the antioxidant re-

Fig. 3. Protective Effect of Baicalin on H_2O_2 Induced Cell Death in NIH3T3 Fibroblast

Cells (3×10^4 /well) grown either with or without Baicalin for 24 h were treated with 0.5 mM H_2O_2 for 2 h. The cell bioavailability was determined by the MTT staining method. Data are the means $\pm$ S.D. of 6 cell cultures. $p < 0.05$.

sponsive element,²⁴⁾ because Baicalin pre-treated NIH3T3 cells indeed enhanced resistance against H_2O_2 abuse (Fig. 3). The present experiments suggest that flavonoids in plants might act to modify biological responses which enhance the cellular potential for repairing oxidatively damaged DNA.

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