Human gastric cells resistant to (−)-epigallocatechin gallate show cross-resistance to several environmental pollutants

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Abstract

After a long-term culture in (−)-epigallocatechin gallate (EGCG, 20 μM), a major constituent of green tea, human gastric AGS cells developed 2.2-fold resistance to EGCG. The resistant AGS (AGS-R) cells were cross-resistant to several N-methylcarbamate insecticides, which are among the major control agents for pest insects in Taiwan. The AGS-R cells also showed protective effects against both the cytotoxicity and DNA damage induced by one of the mutagenic derivatives of N-methylcarbamate insecticide, N-nitroso methomyl, which is known to target the mammalian gastric tract. Therefore, acquisition of resistance by AGS cells through chronic exposure to EGCG implies that the tea-drinking habit of the Taiwanese is probably beneficial for the health of the gastric tract. In addition, AGS-R cells were cross-resistant to sodium arsenite and hydrogen peroxide, indicating that tolerance to oxidative stress might play a role in the development of resistance described in this investigation.

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1. Introduction

Tea is the most widely consumed beverage next to water in the world. Its main constituent, (−)-epigallocatechin gallate (EGCG), is biologically active in antioxidant, antiangiogenesis, and antiproliferative assays that are relevant to the prevention of various forms of disease, including cancer (Cooper et al., 2005a,b). Results of epidemiological studies indicating low gastric cancer risk among people who consume large quantities of green tea led to investigations of EGCG’s effect on gastric cancer prevention (Yamane et al., 1995, 1996). In animal experiments with a mouse model, EGCG was reported to inhibit MNNG-induced gastric carcinogenesis (Yamane et al., 1995). When exposed to EGCG, the growth of KATO III, a type of human stomach cancer cell, was reduced and apoptosis developed (Hibasami et al., 1998). Proliferation of KATO III and three other human gastric cell lines was found to be inhibited by EGCG in dose-dependent manners (Horie et al., 2005). In human gastric AGS cells, EGCG showed an anti-invasive effect by controlling MMP expression through the suppression of MAPK and AP-1 activation (Kim et al., 2004). EGCG also has significant protective effects against Helicobacter pylori-induced gastric cytotoxicity via interference with TLR-4 signaling. On the whole, EGCG has profound medicinal benefits to human gastric cells. A previous study using a mouse model indicated that the protocol for multiple administration enhanced incorporation of EGCG by 4–9 times in most organs (Suganuma et al., 1998). The phenomenon was termed the “Fujiki–Suganuma effect”, and led to the proposal for the multiple administration of EGCG in order to increase the concentration in cells and in vivo (Fujiki, 2005). Due to the fact that tea drinking is an everyday habit among Taiwanese people, the “Fujiki–Suganuma effect” inspired us to investigate the long-term effect of EGCG...
on chemoprevention in human gastric cells. In this report, we describe the development of a cytoprotective effect against several environmental pollutants in human gastric AGS cells through long-term culture with EGCG. Most of the environmental pollutants included were N-methylcarbamate insecticides. AGS is a permanent cell line, and clones were established from an untreated patient with gastric carcinoma (Barranco et al., 1991). N-Methylcarbamate insecticides are major pest control agents in this country and have been reported to turn into mutagenic N-nitroso metabolites which act on mammal gastric tissue as the target organ in animal experiments (Ljinsky, 1992).

2. Materials and methods

2.1. Cells

The human AGS gastric carcinoma cell line, originally purchased from American Type Culture Collection (ATCC; Rockville, MD, USA), was cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM l-glutamine (GIBCO BRL), and was kept at 37 °C in the dark in a humidified 5% CO2 incubator. EGCG-resistant cells (AGS-R cell) were developed by continuously culturing AGS cells in complete RPMI 1640 medium containing 20 μg/ml EGCG (Sigma Chemical, St. Louis, MO, USA) for 50 passages.

2.2. Chemicals

Sodium arsenite and hydrogen peroxide were products of Sigma Chemical. N-methylcarbamate insecticides were purchased from Chem Service (West Chester, PA, USA) and included aldicarb (2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl) oxime, CAS 116-06-3), carbaryl (1-naphthyl N-methylcarbamate, CAS 63-25-2), methomyl (S-methyl-N-(methylcarbamoyl)thioacetimidate, CAS 16752-77-7), and propoxur (O-isopropoxyphenyl methyl carbamate, CAS 114-26-1). The N-nitroso derivative of N-methyl methomyl insecticide was prepared according to the method described by Blevins et al. (1977). Five grams of the insecticide was dissolved in 15 ml glacial acetic acid (E. Merck, Darmstadt, Germany). Five milliliters of double-distilled water (ddH2O) was added. The mixture was then cooled on ice, and 6 g of sodium nitrite (Merck) was added with gentle shaking. The solution was kept cold for 1 h and then at room temperature for another hour. Fifty milliliters of ether was added, followed by 50 ml ddH2O. In order to remove the acetic acid, the solution was shaken four times with 50 ml ddH2O. The yellow ether layer was thus separated off and dried with anhydrous sodium sulfate. The solution was kept cold for 1 h before being centrifuged at 15,000 rpm. Seventy microliters of 1% low-melting-point agarose was added, followed by 50 ml ddH2O with floating ice. The coverslips were removed. Seventy microliters of 1% low-melting-point agarose was applied as the third layer of agarose. The gel was covered with a coverslip and placed on top of ice for 5 min. After removing the coverslip, the slides were immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM sodium ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris (with the pH adjusted to 10.0 with NaOH), with 1% N-lauroylsarcosine, 1% Triton X-100, and 10% dimethyl sulfoxide added immediately before use) at 4 °C overnight. Slides were denatured in an electrophoresis tank containing 0.3 M NaOH (pH 13.4) and 1 mM sodium EDTA for 20 min, with the level of the solution 2–3 mm above the gel. Electrophoresis was carried out at 25 V and 300 mA (with the current adjusted to the buffer volume) for 30 min. Slides were briefly washed in ddH2O, blotted, and then transferred to 0.4 M Tris–HCl at pH 7.5. The slide was stained with YOYO-1 (Molecular Probes, Eugene, OR, USA; 50 μl of a 20-μM solution prepared in glycerol/0.4 M Na2EDTA, at pH 7.4 (50/50), containing 0.1% S-hydroxyquinoline), and a coverslip was applied. The comets were observed under a fluorescence microscope (shortwave pass filter 450–490 nm, chromatic beam splitter 510 nm, longwave pass filter 520 nm). The image of 100 cells per treatment was recorded with a digital camera (DCS-420; Kodak, Rochester, NY, USA). Migration of DNA from the nucleus in each cell was expressed by the parameter of the tail moment and measured with Comet Assay III software (available at www.perceptive.co.uk).

2.3. Cell survival assays

A method using UV absorption as an approximation for cell number as proposed by Chang (1991) was used to analyze the survival of AGS and AGS-R cells when exposed to the test chemicals. Briefly, 1 × 105 cells were plated onto each 35-mm Petri dish for overnight incubation. After treatment with chemicals in a series of different doses for specified periods, cells were trypsinized and collected in a centrifuge tube, washed twice with cold calcium magnesium-free phosphate-buffered saline (PBS), extracted twice with cold methanol:acetic acid (3:1) to remove the free nucleosides and nucleotides, and solubilized at 37°C with a 0.2 N NaOH solution at 105 cells/ml for 24 h. The absorption at 260 nm for each cell solution was determined using a Hitachi U2000 spectrophotometer. The percent survival with each treatment was estimated by dividing the absorbance of the treatment by that of the control.

2.4. DNA damage assay

The procedure for the single-cell alkaline electrophoresis (the comet assay) followed that described previously (Liu and Jan, 2000). Briefly, agarose with a normal melting point at 1% in PBS was incubated in a water bath at 65 °C. Microscope glass slides were placed on a 60 °C hot plate. One hundred microliters of agarose gel solution was dropped onto a slide, and a coverslip was immediately applied to cover it. Slides were placed on top of ice to allow the gel to set. The coverslips were removed by dipping the slides into ddH2O with floating ice. Low-melting-point agarose at 1.2% in PBS was incubated in a water bath set at 40 °C. Fifty microliters of a cell suspension containing 2 × 105 cells in PBS and 250 μl of an agarose solution were thoroughly mixed, and 65 μl of the mixture was pipetted onto the slide. After applying a coverslip, the slide was placed on top of ice for 5 min. The slides were dipped in ddH2O with floating ice, and the coverslips were removed. Seventy microliters of 1% low-melting-point agarose was applied as the third layer of agarose. Again, the gel was covered with a coverslip and placed on top of ice for 5 min. After removing the coverslip, the slides were immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM sodium ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris (with the pH adjusted to 10.0 with NaOH), with 1% N-lauroylsarcosine, 1% Triton X-100, and 10% dimethyl sulfoxide added immediately before use) at 4°C overnight. Slides were denatured in an electrophoresis tank containing 0.3 M NaOH (pH 13.4) and 1 mM sodium EDTA for 20 min, with the level of the solution 2–3 mm above the gel. Electrophoresis was carried out at 25 V and 300 mA (with the current adjusted to the buffer volume) for 30 min. Slides were briefly washed in ddH2O, blotted, and then transferred to 0.4 M Tris–HCl at pH 7.5. The slide was stained with YOYO-1 (Molecular Probes, Eugene, OR, USA; 50 μl of a 20-μM solution prepared in glycerol/0.4 M Na2EDTA, at pH 7.4 (50/50), containing 0.1% S-hydroxyquinoline), and a coverslip was applied. The comets were observed under a fluorescence microscope (shortwave pass filter 450–490 nm, chromatic beam splitter 510 nm, longwave pass filter 520 nm). The image of 100 cells per treatment was recorded with a digital camera (DCS-420; Kodak, Rochester, NY, USA). Migration of DNA from the nucleus in each cell was expressed by the parameter of the tail moment and measured with Comet Assay III software (available at www.perceptive.co.uk).

2.5. Reduced glutathione (GSH) assay

GSH levels were measured according to a fluorometric method described by Cohn and Lyle (1966). Before each assay, a standard curve with at least five distinct concentrations of GSH was prepared by dissolving GSH to cover the range of 20–100 μmol/l in the reaction medium in 5% metaphosphoric acid. Twenty microliters of the GSH solution was added into 2 ml of ddH2O, then mixed with 0.5 ml sodium PBS (1 M, pH 8.0) and 0.1 ml O-ophthaldehyde (0.1%, w/v). The mixture was incubated at room temperature in the dark before reading its absorbance at 420 nm (with excitation at 350 nm) using a fluorescence spectrophotometer (Hitachi F4500). For the assay, cells were washed with PBS, trypsinized with 0.25% Trypsin-EDTA, and centrifuged at 300g for 5 min. The cell lysate was mixed with 200 μl chilled ddH2O and 50 μl 25% metaphosphoric acid before being centrifuged at 15,000g at 4 °C for 15 min. Twenty microliters of the supernatant was added to 2 ml of ddH2O, and GSH activity was measured as described above for preparation of the standard curve.

3. Results

In order to verify the method used to assay the cell survival in this study, six different numbers of AGS cells in a series ranging from (0.5 to 8) × 105 each were sampled to investigate the correlation between the cell number and
the relative absorption at UV 260 nm. A significant linear correlation of $r^2 = 0.99$ was obtained (Fig. 1). This indicates that the method using UV 260 nm absorbance we adapted from Chang (1991) is capable of giving an unbiased estimation of cell numbers during the survival assays.

At concentrations $\leq 10 \mu M$, EGCG was not toxic to the human gastric cell line, AGS, in a 3-day treatment protocol (Fig. 2). When concentrations approached $20 \mu M$, the cytotoxicity of EGCG abruptly increased, leading to a decrease in the survival of AGS cells to about 30% of the control group. EGCG at concentrations $\geq 30 \mu M$ was extremely cytotoxic to AGS cells, and in most cases, no cells survived. AGS cells which survived the EGCG treatment at $20 \mu M$, after being continuously cultured in EGCG-containing medium ($20 \mu M$) for over five passages, turned out to be a novel population which vigorously proliferated in the culture containing $20 \mu M$ EGCG. Those EGCG-resistant cells were kept in the same EGCG-containing medium and routinely subcultured twice a week up to the 50th passage before being cryostored in liquid nitrogen and named AGS-R for further experimental use in this study. The dose–response curve shown in Fig. 2 indicates that treatment with $20 \mu M$ EGCG was not cytotoxic to AGS-R cells. AGS-R cells also showed a higher survival rate than parental AGS cells at higher concentrations of EGCG, including 30 and $40 \mu M$ (Fig. 2). However, they became equally susceptible as the concentrations of EGCG approached $50 \mu M$. The 50% lethal dose ($LD_{50}$) values of EGCG to AGS and AGS-R, calculated using CalcuSyn (vers. 1.1), were 19 and $30 \mu M$, respectively, indicating that an overall 2.2-fold higher EGCG-resistance was developed in AGS-R cells.

AGS-R cells were found to be cross-resistant to several major $N$-methyl carbamate insecticides (Fig. 3), including aldicarb, carbaryl, methomyl, and propoxur, which are among the most commonly used insecticides in farming and domestic activities in Taiwan. According to their $LD_{50}$ values obtained from a 24-h treatment protocol, there was a 2.7-fold increase in the development of resistance to aldicarb, which was the most profound among the four insecticides assayed. The resistance level of AGS-R cells to methomyl and propoxur, also estimated from the $LD_{50}$ values, was twice that of parental AGS cells. The development of resistance to carbaryl was the least among four insecticides, nevertheless it showed a 1.5-fold increase in AGS-R cells.

In addition to the parental $N$-methylcarbamate insecticide, AGS-R cells developed cross-resistance to the mutagenic $N$-nitroso derivatives of methomyl. The $LD_{50}$ values of $N$-nitroso methomyl in AGS and AGS-R cells, obtained from a 2-h treatment protocol, were 0.27 and 4.10 $\mu g/ml$, respectively, indicating that AGS-R cells are 15 times more resistance to $NO$-methomyl (Fig. 4). The genotoxicity of $N$-nitroso $N$-methylcarbamate insecticides to mammalian cells has previously been reported (Wang et al., 1998a,b; Yoon et al., 2001). Like most of the $N$-nitroso compounds, they are potent, direct-acting mutagens and carcinogens, inducing DNA strand breaks. We therefore investigated the DNA strand breaks induced by $N$-nitroso methomyl in AGS and AGS-R cells using the COMET assay with single-cell electrophoresis techniques. In treatments with $N$-nitroso methomyl at 1 and $2 \mu g/ml$ for 2 h, the median values of the tail moment in AGS-R cells decreased 2.2 and 1.5 folds when compared to those in parental AGS cells. The overall distribution range of the tail moment also indicated that far more serious DNA damage was induced in AGS cells than in AGS-R cells (Fig. 4). Therefore, AGS-R cells were also resistant to the genotoxicity induced by $N$-nitroso methomyl.

AGS-R cells not only developed cross-resistance to insecticides but also to ROS-generating substances...
Fig. 3. Cytotoxicity of $N$-methyl carbamate insecticides to human gastric AGS (open circles) and EGCG-resistant AGS-R (solid circles) cells.

Fig. 4. Cytotoxicity (a) and DNA damage (b) induced by $N$-nitroso methomyl to human gastric AGS (circles) and EGCG-resistant AGS-R (rectangles) cells.

Fig. 5. Cytotoxicity of sodium arsenite (a) and hydrogen peroxide (b) to human gastric AGS (open circles) and EGCG-resistant AGS-R (solid circles) cells.
including sodium arsenite and hydrogen peroxide. The most profound difference in susceptibility to sodium arsenite between these two cell lines occurred for treatments in the concentration range of from 4 to 16 μM. The LD_{50} values of sodium arsenite to AGS and AGS-R cells were 2.26 and 9.80 μM, respectively, in a 24-h treatment protocol (Fig. 5). AGS-R cells therefore were four times more resistant to sodium arsenite than were parental AGS cells. Similarly, AGS-R cells showed 1.5-fold higher resistance to hydrogen peroxide than AGS cells in the 1-h treatment protocol (Fig. 5).

In addition, ROS compounds have frequently been reported to be targeted by intracellular reduced glutathione (GSH) (Townsend et al., 2003). Intracellular GSH levels were correlated with arsenic resistance (Lee et al., 1989) and, when depleted, caused the sensitization of cells to arsenic compounds (Shimizu et al., 1998). Furthermore, stimulation of intracellular GSH in AGS cells was found to enhance the protective effect against damage induced by sodium taurocholate (Rodriguez et al., 2005a,b). Therefore, intracellular GSH levels were compared between AGS and AGS-R cells in order to elucidate the mechanism of resistance of sodium arsenite as well as to other ROS compounds. The basal GSH level found in the parental AGS was around 10 nmoles/million cells, which was higher than some previous studies (Filomeni et al., 2005; Rodriguez et al., 2005a), however was close to those of Shirin et al. (2001). In AGS-R cells, the intracellular GSH level was seven times as high as in parental AGS cells (Fig. 6).

4. Discussion

N-Methylcarbamate insecticides are major pest insect control agents in Taiwan as well as many other countries in subtropical and tropical areas. In addition to their acute toxicity to cholinesterase, several N-methylcarbamate insecticides inhibit gap junctional intercellular communication of mammalian cells in culture (Wang et al., 1998a,b). This implies that N-methylcarbamate insecticides have the potential to act as tumor-promoting agents (Yotti et al., 1979). Under physiological conditions of the stomach of being mildly acidic and 37 °C, N-methylcarbamate insecticides are converted into mutagenic N-nitroso derivatives (Blevins et al., 1977). N-nitroso N-methylcarbamate insecticides were previously shown to induce sister-chromatid exchange, chromosome aberrations, gene mutations, and preneoplastic transformation in mammalian cells (Wang et al., 1998a,b). As a result, the potentially hazardous effects of N-methylcarbamate insecticides represent a significant issue to human health. Data from animal experiments indicated that the gastric tract is the major target of N-nitroso N-methylcarbamate insecticides (Lijinsky, 1992). Therefore, in inhabitants of area where insecticide application is extensive, endogenous N-nitrosation of N-methylcarbamate insecticides in the digestive tract might play a significant role in the etiology of human gastric cancer. Our findings that human gastric cells gain resistance to cytotoxicity and DNA-damaging effects of N-methylcarbamate and its N-nitroso derivatives through chronic exposure to EGCG is an important benefit to human gastric health.

According to government’s statistics, people in Taiwan consumed over 40,000 ton tea in the year of 2005. Based on the tea polyphenol composition estimated by Fujiki (2005), there were approximately 457 μg EGCG consumed per day by each person. Therefore in countries such as Taiwan where tea is the most common daily beverage second only to water, the human gastric tract is presumably affected by tea drinking. This is because the gastric tracts of those who habitually drink tea would have direct contact with the tea solution, usually in high concentrations. Preventive effects of tea polyphenols on the human gastric tract were comprehensively reviewed recently (Koo and Cho, 2004). Evidence from epidemiological and case-control studies showed that tea consumption and gastric cancer risk are inversely related in Oriental countries including Japan, China, and Taiwan (Yang and Wang, 1993). An investigation in the area of Yangzhong, China additionally revealed that green tea drinking retarded the progression of chronic gastritis, a disease usually leading to the intestinal type of stomach cancer (Setiawan et al., 2001). Tea consumption has also been reported to decrease the infection incidence of Helicobacter pylori, a causative microorganism in gastric ulcers and carcinogenesis (Yee et al., 2002). An investigation into its mechanism disclosed that EGCG protected human gastric cells from H. pylori-induced gastric cytotoxicity through interference with TLR-4 glycosylation (Lee et al., 2004). In human gastric AGS cells, EGCG inhibited TPA-induced invasiveness through the reduction of MMP-9’s transcriptional activity (Kim et al., 2004). This implies that EGCG might be able...
to retard the metastasis of gastric cancer. To sum up, EGCG is a versatile chemoprevention agent with different modes of action. Among previous studies, the antioxidant, antiangiogenesis, and antiproliferation effects are most extensively referred to Cooper et al. (2005a,b) and Fujiki (2005). The findings in this article, on the other hand, demonstrate that through the development of cellular resistance, EGCG is cytoprotective against the adverse effects of gastric-targeting insecticides.

Human exposure to N-nitroso compounds is of particular significance in the etiology of gastric cancer (Mirvish, 1995). Risk of human gastric cancer is increased by endogenously formed N-nitroso compounds (Palli et al., 2001). Tea extracts, on the other hand, are known to efficiently inhibit N-nitration in the human gastric tract (Tanaka et al., 1998). The ability of tea to inhibit endogenous N-nitrosation is positively correlated to its polyphenol contents (Wang and Wu, 1991). In the results of the COMET assay shown in this article, the tail moment induced by N-nitroso methomyl was significantly smaller in human gastric AGS cells that had been chronically cultured in EGCG-containing medium. In addition to inhibiting endogenous N-nitrosation, we demonstrated that EGCG directly decreased the cytotoxicity and DNA damage induced by N-nitroso insecticides.

In addition to insecticides, AGS-R cells also developed cross-resistance to sodium arsenite. Although arsenic compounds have not previously been documented as a potential gastric-targeting agent, it is a widely distributed pollutant in nature and is released into the environment through industrial processes and agricultural usage. In Taiwan, long-term ingestion of arsenic-contaminated artesian water plays a significant role in the etiology of blackfoot disease (Chen and Wu, 1962). Chronic exposure to arsenic is associated with increased risks for cancers of the skin, lung, liver, and prostate (Chen et al., 1985, 1990, 1992; Chiou et al., 1995). Development of cellular resistance to sodium arsenite through chronic exposure to EGCG, consequently, is significant for human health as well.

Compared with N-methylcarbamate insecticides, sodium arsenite is a much more thoroughly studied environmental pollutant, especially its mechanism of cytotoxicity. The cross-resistance of AGS-R cells to sodium arsenite, therefore, offers clues for exploring the mechanism of resistance to N-methylcarbamate insecticides, about which limited knowledge can be found in the literature. Most previous investigations recognized that oxidative stress plays a significant role in arsenic-induced toxicity (Hei and Filipic, 2004; Huang et al., 2004; Shi et al., 2004). One of the major effects of oxidative stress, lipid peroxidation, on the other hand, has been reported to be involved in the molecular mechanisms of toxicity of N-methylcarbamate insecticides, including aldicarb (Yarsan et al., 1999), carbaryl (Soderpalm-Berndes and Onfelt, 1988), carbofuran (Milatovic et al., 2005), and propoxur (Banerjee et al., 1999; Seth et al., 2000). Accordingly it is possible that the tolerance to oxidative stress is among the major mechanisms responsible for the resistance of AGS-R cells to N-methylcarbamate insecticides. The outcomes that AGS-R cells are cross-resistant to hydrogen peroxide and possess higher endogenous GSH levels confirm the assumption.

As to the mechanisms of resistance to N-nitroso insecticide derivatives, there are some controversies. N-nitroso carbaryl has been found to inhibit microsomal lipid peroxidation (Beraud et al., 1989). MNNG, a major N-nitroso compound, on the contrary, was reported to raise the production of thiobarbiturate (TBA)-reactive species in hepatocytes (Reitman et al., 1988), to enhance lipid peroxidation in the stomach of male Wistar rats (Arivazhagan et al., 2000; Subapriya et al., 2003) and of male Swiss albino mice (Subapriya et al., 2004), and to produce hydroxyl radicals in gastric cancer cells (Mikuni and Tatsuta, 2002) and in hamster and human cells (Labaj et al., 2003). In addition, cross-resistance of oxidatively stressed cells to other toxic agents including MNNG has been observed (Crawford and Davies, 1994). An adaptive response to oxidative stress was the explanation of the researchers for the occurrence of cross-resistance. However, in our results, an adaptive response is probably unable to explain the development of resistance or cross-resistance in AGS-R cells. Since the dose of EGCG we used for the chronic culture originally killed about 70% of the population of parental AGS cells, the possibility that the resistance developed through selection by EGCG cannot be excluded. In view of the fact that the long-term culture with EGCG enhanced the intracellular antioxidant capacity in our results, selection forces might be attributed to the prooxidant activity of EGCG, which has been frequently associated with its cytotoxicity in previous studies (Lambert et al., 2005).

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