Induction of Adaptive Response and Enhancement of PC12 Cell Tolerance by 7-Hydroxycholesterol and 15-Deoxy-Δ^{12,14}-Prostaglandin J₂ through Up-regulation of Cellular Glutathione via Different Mechanisms*

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Increasing evidence suggests an adaptive response induced by reactive oxygen species and other physiologically existing oxidative stimuli. We have recently reported that a variety of lipid peroxidation products at sublethal concentrations could induce adaptive response and enhance PC12 cell tolerance, although the detailed underlying molecular mechanisms have not been clearly clarified.

In the present study, we found that both 7-hydroxycholesterol (7-OHCh) and 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) at sublethal concentrations significantly increased the cellular GSH as well as the enzyme activity of glutamate-cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis. Depletion of cellular GSH by buthionine sulfoximine completely abolished the adaptive response. Interestingly, treatment with 15d-PGJ₂ significantly increased the gene expression of both subunits of GCL in an NF-E2-related factor 2 (Nrf2)-dependent manner, whereas neither 7-OHCh induced any considerable changes on the GCL gene expression nor did the Nrf2-small interfering RNA treatment exert any appreciable effects on the GSH elevation and subsequent adaptive response induced by 7-OHCh. These results demonstrate that the adaptive response induced by both 7-OHCh and 15d-PGJ₂ is mediated similarly through the up-regulation of GSH but via different mechanisms.

The generation of reactive oxygen species (ROS) and subsequent oxidative modification of biomolecules such as phospholipids are inevitable in aerobic organisms. Lipid peroxidation has a Janus face. It has been implicated in a variety of pathological events such as atherosclerosis, ischemia-reperfusion injury, cardiovascular diseases, and neurodegenerative diseases (1), but at the same time, the lipid peroxidation products, like other ROS, have now been considered to play an important role in cellular signal transduction. It may directly damage and impair the fine structure and function of biomembranes and may also modify biologically essential lipids, proteins, and nucleic acids. On the other hand, such oxidative stress may also stimulate the defense network, thereby triggering the adaptive response.

Oxysterols are oxygenated derivatives of cholesterol that are important as intermediates or end products in cholesterol excretion pathways (2) and have been shown to be involved in various diseases such as the atherosclerotic lesions (3). Among those physiologically occurring oxysterols, 7-hydroxycholesterol (7-OHCh) suggests an increase of lipid peroxidation in vivo and has been successfully detected as an in vivo oxidative stress marker (4).

Prostaglandins of the J series (PGJs) are cyclopentenones synthesized from arachidonic acid via enzymatic conversion by cyclooxygenase and prostaglandin D₂ synthase followed by nonenzymatic dehydration from prostaglandin D₂ to a series of PGJs, including 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) (5). It has been widely accepted that 15d-PGJ₂ can react with nucleophiles such as the free sulfydryl of GSH and cysteine residues in cellular proteins through the Michael addition reaction due to its reactive α,β-unsaturated carbonyl functional group in the cyclopentenone ring. Thus, it has been suggested that 15d-PGJ₂ could act as a potential activator of NF-E2-related factor 2 (Nrf2) and induce the expression of phase II detoxification enzymes (6–8).

The role of GSH in the protection of cells against oxidative stress and other xenobiotic compounds has been well established (9). The intracellular content of GSH is a function of the balance between consumption, regeneration, and synthesis. Although considerable evidence shows that GSH synthesis is regulated by glutamate cysteine ligase (GCL) activity, cysteine availability, and GSH feedback inhibition (10, 11) together, it should be emphasized that in most cases, the elevation of GSH is due principally to the de novo synthesis, a consequence of the increased enzyme activity of GCL, the rate-limiting enzyme of GSH biosynthesis (12). GCL is a heterodimer that can be dissociated under denaturing conditions into a catalytic subunit (GCLC) and a modulatory subunit (GCLM) (13). It has been widely accepted that both GCLC and GCLM genes are antioxidant response elements (AREs)-derived, and they both can be transcriptionally regulated by Nrf2, a central transcription factor that interacts with the ARE to activate gene transcription constitutively or in response to an oxidative stress signal (14–16).

The adaptive response induced by ROS and other physiologically existing oxidative stimuli has received increased attention (17–22). We...
have recently reported that the adaptive response induced by 4-hydroxynonenol (4-HNE), one of the end products of lipid peroxidation, was critically mediated through the induction of thioredoxin reductase 1 (TR1) via activation of Nrf2 (21). We have also reported that a variety of other lipid peroxidation products could induce adaptive response and enhance PC12 cell tolerance against the subsequent oxidative stress (22), but the detailed underlying molecular mechanisms have not been clearly clarified. In the present study, we found that the adaptive response induced by 7-OHCh and 15d-PGJ2 was similarly mediated by the elevated GSH, whereas the detailed mechanisms for the GSH elevation were different.

**EXPERIMENTAL PROCEDURES**

**Materials**—Authentic 7a- and 7β-OHCh were obtained from Steraloids Inc., and 15d-PGJ2 was a product from Calbiochem. NAPDH, reduced GSH, and oxidized GSSG were purchased from Nacalai, Kyoto, Japan. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Dojindo Kumamoto, Japan. 6-Hydroxydopamine (6-OHDA) was from Sigma. Antibodies against Nrf2 and actin were from Santa Cruz Biotechnology. All other chemicals were of the highest quality commercially available.

**Cell Culture and Determination of Cell Viability**—PC12 cells were routinely cultured in Dulbecco’s modified Eagle’s medium/F-12 medium with 1-glutamine, sodium bicarbonate, and pyridoxine hydrochloride, supplemented with 10% fetal bovine serum and 5% horse serum. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2.

For determination of the cell viability, the conventional MTT reduction assay was used. The treated cells were incubated with 0.5 mg/ml MTT at 37 °C for 1 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2 by volume) and mixed with a pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Thermo LabSystems, Helsinki, Finland).

**Measurement of Cellular GSH Contents**—Intracellular GSH contents, including both reduced and oxidized forms, were determined enzymatically by a modified 5,5′-dithiobis-(2-nitrobenzoic acid) assay according to the method described elsewhere (23).

**Preparation of the Cytosolic Fractions and Analysis of GCL Activity**—The cytosolic fractions were prepared for the following GCL enzyme activity assay, as described previously (24). GCL activity was determined using the method reported elsewhere (25), with slight modification. Reaction mixture (0.1 M Tris-HCl buffer, pH 8.2, 1 ml) was added to the culture medium (3:2 by volume) and mixed with a pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Thermo LabSystems, Helsinki, Finland).

**RESULTS**

**Adaptive Response Induced by 7-OHCh and 15d-PGJ2 in PC12 Cells**—To explore the possible adaptive response, the cytotoxicity induced by these compounds was first investigated in PC12 cells. As shown in Fig. 1A, all of these three compounds, 7α-OHCh, 7β-OHCh, and 15d-PGJ2, induced significant cell death at high concentrations. Pretreatment of PC12 cells with sublethal concentrations of each compound (7-OHCh, 20 μM; 15d-PGJ2, 2.5 or 7.5 μM) significantly protected cells against the oxidative stress induced by 6-OHDA (Fig. 1B), which has been demonstrated to induce oxidative damage in neural system and implicated in neurodegenerative disorders (27).

**Effect of 7-OHCh and 15d-PGJ2 on the Intracellular GSH Contents**—This interesting adaptive phenomenon led us to explore the possible underlying molecular mechanisms. Since we have recently observed the pivotal role of TR1 in 4-HNE-induced adaptive response (21), we then studied the possible involvement of TR1 in the adaptive response induced by 7-OHCh and 15d-PGJ2. However, both 7-OHCh and 15d-PGJ2 failed to induce any significant variation on the TR1 activity (data not shown). The intracellular GSH is well known to be essential to antioxidative defense (9); we therefore attempted to study the possible involvement of GSH in the adaptive response. Treatment of either 7-OHCh or 15d-PGJ2 at their sublethal concentrations for 24 h significantly increased the total cellular GSH contents (Fig. 2, A and B). These treatments did not cause any considerable changes on the ratio of oxidized GSSG/total GSH, retaining at ~6% without much variation (data not shown). In the present study, we found that the adaptive response induced by 7-OHCh and 15d-PGJ2 was similarly mediated by the elevated GSH, whereas the detailed mechanisms for the GSH elevation were different.
not shown). Both 7α- and 7β-OHCh at 20 μM induced ~1.6-fold GSH increases (Fig. 2A), whereas 15d-PGJ2 at 7.5 μM dramatically enhanced the cellular GSH until ~3-fold (Fig. 2B).

We then investigated the time-dependent induction of GSH by 7α-OHCh and 15d-PGJ2. Interestingly, treatment of either 7α-OHCh (20 μM) or 15d-PGJ2 (7.5 μM) significantly reduced the cellular GSH level to ~80 or 60%, respectively, of the basal level at early time (before 3 h), but the GSH level recovered back thereafter time-dependently, reaching to the final 1.6- or 3-fold, respectively, at 24 h (Fig. 2C).

**Increased GCL Activity and Subsequent GSH Elevation Were Responsible for the Adaptive Cytoprotection Induced by both 7-OHCh and 15d-PGJ2**—The elevation of the intracellular GSH contents should owe primarily to the augmented GCL activity; we therefore investigated the possible increase in the GCL enzyme activity induced by 7α-OHCh and 15d-PGJ2. The GCL activity was rapidly increased for ~20% by 7α-OHCh at 3 h, and it held at the same level until 24 h (Fig. 3A). 15d-PGJ2 time-dependently increased the GCL activity, finally with ~30% elevation at 24 h (Fig. 3A).

To further confirm the role of the increased GCL activity and subsequent GSH elevation in the adaptive response, we attempted to explore the effect of buthionine sulfoximine (BSO), a specific GCL inhibitor widely used in a variety of cell lines (28, 29). Treatment of BSO at even 0.1 μM for 24 h significantly decreased the constitutive GSH level in PC12 cells but without any appreciable cytotoxicity until 10 μM (data not shown). Pretreatment of 0.1 or 1.0 μM BSO together with 7α-OHCh or 15d-PGJ2 effectively attenuated (in the case of 0.1 μM BSO) or completely abolished (in the case of 1.0 μM BSO) the adaptive cytoprotection induced by these oxidative stimuli (Fig. 3B), suggesting a pivotal role of the increased GCL activity and subsequent GSH elevation in the adaptive response.

**Effect of 7-OHCh and 15d-PGJ2 on the Expression of GCL**—In most cases, the increased GCL activity must be due principally to the increased GCL gene expression and subsequent enhancement on the GCL proteins; we then investigated the mRNA expression of GCLC and GCLM by these compounds. As expected, 15d-PGJ2 at 7.5 μM induced significant gene expression of both GCL subunits, which both were induced by around 2.5-fold peaked at 6 h (Fig. 4, A and B). Although the gene expression induced by 2.5 μM 15d-PGJ2 was lesser than that by 7.5 μM, it was still significant (Fig. 4, A and B). Interestingly, both 7α- and 7β-OHCh at 20 μM did not induce any considerable mRNA induction of both GCL genes (Fig. 4, A and B), suggesting the possible different mechanisms mediated for the increased GCL activity and GSH elevation by 7α-OHCh and 15d-PGJ2.
Possible Involvement of Nrf2 in the GSH Elevation and Subsequent Adaptive Response Induced by 7-OHCh and 15d-PGJ2—The transcription factor Nrf2 has been implicated as the central protein that interacts with the ARE to induce the expression of a cluster of phase II enzymes, including GCL (12); we therefore studied the possible involvement of Nrf2 in the GSH elevation and subsequent adaptive response induced by 7-OHCh and 15d-PGJ2. The nuclear Nrf2 level was rapidly accumulated by the treatment of 15d-PGJ2 but not by 7α-OHCh (Fig. 5A). In cells treated with Nrf2-siRNA, the 15d-PGJ2-induced gene expression of GCL was significantly attenuated (Fig. 5, B and C). As a consequence, the enhanced GSH induced by 15d-PGJ2 was markedly decreased by the Nrf2-siRNA treatment (Fig. 5D). However, it only slightly, but not significantly, decreased the 7-OHCh-induced GSH elevation (Fig. 5D). Moreover, in such Nrf2-siRNA-treated cells, 15d-PGJ2 at 2.5 μM failed to induce any adaptive response, whereas both 7α- and 7β-OHCh still induced significant adaptive cytoprotection, although the protective effect was smaller than that in the normal cells (Fig. 5E). 15d-PGJ2 at 7.5 μM also induced adaptive protection in the Nrf2-siRNA-treated cells, but the protective effect was significantly decreased when compared with that in normal cells (data not shown).

**DISCUSSION**

The essential roles of low levels of H2O2 and related ROS in the cell signal transduction and immuno-defense have been widely accepted (31). Accumulating evidence also suggests that low levels of ROS can induce adaptive response and enhance tolerance in cultured cells (17–20) and in animals (32, 33). Likewise, such roles of some other physiological oxidative stimuli or oxidation products in modulating and participating in signal transduction have received increased attention. For
GSH Elevation Mediates 7-OHCh and 15d-PGJ₂ Adaptive Response

FIGURE 5. Possible involvement of Nrf2 signaling pathway in the adaptive response induced by 7-OHCh and 15d-PGJ₂. A, nuclear accumulations of Nrf2 by 15d-PGJ₂, but not by 7α-OHCh. PC12 cells were treated with 7.5 μM 15d-PGJ₂ or 20 μM 7α-OHCh for the indicated times, and nuclear fractions were extracted for Western blotting analysis. B and C, Nrf2-siRNA effectively attenuated the mRNA expression of GCLC (B) and GCLM (C) induced by 15d-PGJ₂. PC12 cells were pretreated with Nrf2-siRNA or control RNA sample for 24 h followed by the treatment of 15d-PGJ₂ for an additional 9 h. Total RNA was isolated and subjected to real-time PCR analysis. D, Nrf2-siRNA effectively attenuated the GSH elevation induced by 15d-PGJ₂ but without any considerable effect on that by 7-OHCh. PC12 cells were pretreated with Nrf2-siRNA or control RNA sample for 24 h followed by the treatment of 15d-PGJ₂ or 7-OHCh for an additional 24 h. Cells were then harvested for GSH analysis. *, significant difference (p < 0.05). NS, nonsignificant difference. E, Nrf2-siRNA significantly attenuated the adaptive cytoprotection induced by 15d-PGJ₂ but with less effect on that induced by 7-OHCh. PC12 cells were pretreated with Nrf2-siRNA for 24 h followed by treatment with or without these compounds for another 24 h. Cells were then challenged with 6-OHDA for an additional 24 h. Cell viability was determined by MTT assay.

instance, 4-HNE, an α,β-unsaturated aldehyde formed by the reaction of reactive oxygen or nitrogen species with arachidonic acid in cellular membranes during oxidative stress, has now been recognized as a signaling molecule at low concentrations and considered to bridge the fields of oxidative stress and redox signaling (21, 34, 35). Similarly, cyclopentenone prostaglandins, which were widely known as apoptogens in a variety of cell types (36, 37), now have been reported to show protective effect at low concentrations via different mechanisms (6, 28–30). We have recently found that a variety of lipid peroxidation products induced a general adaptive response in cell cultures (22), and we have successfully demonstrated that the 4-HNE-induced adaptive response in PC12 cells was primarily mediated through induction of TR1 via activation of Nrf2 (21); however, the detailed molecular mechanisms mediated for other compounds have not been fully understood. In the present study, we clearly demonstrated that the adaptive response by both 7-OHCh and 15d-PGJ₂ in PC12 cells is mediated similarly through the up-regulated GSH but via different mechanisms.

Cellular GSH is found in the millimolar range in most cells and is well known to be essential to antioxidative defense, regulation of the cell cycle, and gene expression (9). The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two ATP-dependent enzymes, the rate-limiting GCL and GSH synthetase (10, 11). In most cases, the elevation of GSH is due principally to the de novo synthesis, and this increased de novo synthesis is owed primarily to the increased synthesis of GCL subunits through a combination of increased transcription and mRNA stability. Genetic sequence analysis and studies with reporter constructs have revealed that both GCLC and GCLM promoters contain many potential cis-acting elements, including consensus recognition sites for binding of activator protein-1 (AP-1), activator protein-2, nuclear factor κB (NF-κB), and the predominant ARE, with which Nrf2 reacts, thereby inducing the expression of phase II enzymes (12). Due to its reactive α,β-unsaturated carbonyl functional group, 15d-PGJ₂, is able to react through the Michael addition reaction with cellular DNA and proteins, including Kelch-like ECH-associated protein 1 (Keap1), the cytoplasmic inhibitor of Nrf2. This modification of Keap1 results in the liberation and activation of Nrf2, which then translocates into the nucleus and induces the expression of detoxification enzymes. In our present work, we clearly demonstrated the involvement of Nrf2 signaling pathway in the 15d-PGJ₂-induced GSH elevation and subsequent adaptive response. Nrf2 was rapidly accumulated in the nucleus by the treatment of 15d-PGJ₂, which bound to the ARE, thereby up-regulating the expression of detoxifying enzymes, including GCLC and GCLM. It might be noteworthy that the treatment of PC12 cells with Nrf2-siRNA only significantly attenuated, but not completely abolished, the 15d-PGJ₂-induced GCL expression and subsequent GSH elevation, suggesting that other transcriptional factors such as AP-1 or NF-κB may also contribute together in regulation of the GCL gene expression in PC12 cells.

The effect of BSO demonstrated the pivotal role of the increased GCL activity and GSH elevation in the adaptive response, although it could not completely rule out the role if minor, of other antioxidants or other antioxidative enzymes. It might be interesting to note that we have recently reported that 4-HNE-induced adaptive response in PC12 cells was mediated critically through TR1 expression via activation of Nrf2.
(21), whereas in the present study, we demonstrated that the 15d-PGJ2-induced adaptive response was primarily through the Nrf2-mediated GSH elevation. There are probably no fundamentally different mechanisms that 4-HNE and 15d-PGJ2 should have different pathways for inducing the adaptive tolerance in the same cell line. By activating the same Nrf2 signaling pathway, the induction of different detoxifying enzymes may be specific to the different cell types and tissues but should not be specific to the stimulations. The difference in the response pathways in our studies, TR1 for 4-HNE and GSH for 15d-PGJ2, was probably due to the different concentrations used and the different levels of Nrf2 activation. We have also analyzed the time-dependent GSH variation by 4-HNE and found that 4-HNE even at adaptive concentration (15 μM) almost completely depleted the cellular GSH at an earlier time period (data not shown). Thus, although 4-HNE induced marked GCL gene expression (data not shown), the final GSH level at 24 h was not significantly increased when compared with the basal level. On the other hand, 15d-PGJ2 at 7.5 μM did not induce as much TR1 expression as 4-HNE at 15 μM did (data not shown). These findings make it reasonable that the adaptive response induced by 15d-PGJ2 or 4-HNE was mediated through the enhanced GSH or TR1, respectively, which were both induced via activation of Nrf2.

Both 7α- and 7β-OHCh did not induce any considerable expression of GCL, whereas they increased the GCL activity and subsequently enhanced the GSH level, suggesting that other possible mechanisms mediated the enzymatic activation of GCL. It has been widely accepted that, in addition to the increased GCL expression, other factors that stimulate cysteine uptake (38, 39) or attenuate the GSH feedback inhibition (40) would also generally enhance the intracellular GSH concentrations. There is recent evidence that the GCL activity can also be modulated by phosphorylation or nitrosylation (41, 42). It might be noteworthy that cholesterol-5β,6β-epoxide, another cholesterol oxidation product that also induced adaptive response in PC12 cells (22), did not cause any appreciable variation on the cellular GSH level (data not shown), suggesting that the increased enzyme activity of GCL by 7-OHCh was not due to the cholesterol structure. It should be also interesting to note that, similar to the results of 7-OHCh, we have recently found that U0126, a pharmacological inhibitor of extracellular signal-regulated protein kinase, also significantly increased the enzyme activity of GCL and subsequently enhanced the GSH level but without any considerable induction of GCL genes in PC12 cells as well.3 At the present stage, we could not clearly clarify whether the enzymatic activation of GCL by 7-OHCh or U0126 was due to some of the above mentioned mechanisms or some other unknown ones. The further investigation of these mechanisms should provide some constructive information for increasing the GCL activity and consequently enhancing the cellular resistance against oxidative stress.

In conclusion, the present study represents an effort to demonstrate that the adaptive response induced by both 15d-PGJ2 and 7-OHCh in PC12 cells was mediated similarly through the elevated GSH contents but via different molecular mechanisms. Treatment of PC12 cells with 15d-PGJ2 leads to a rapid nuclear translocation of Nrf2, which turns up-regulate the GCL genes, thereby increasing the cellular GSH contents. On the other hand, treatment of 7-OHCh results in an increased GCL enzyme activity and also leads to the elevation of cellular GSH but with neither activation of Nrf2 nor subsequent gene expression of GCL. The adaptive response induced by these compounds eventually enhances the cell tolerance and finally protects cells against the forthcoming oxidative stress.

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