Electrophiles and reactive oxygen species have been implicated in the pathogenesis of many diseases. Transcription factor Nrf2 was recently identified as a general regulator of one defense mechanism against such havoc. Nrf2 regulates the inducible expression of a group of detoxication enzymes, such as glutathione-S-transferase and NAD(P)H:quinone oxidoreductase, via antioxidant response elements. Using peritoneal macrophages from Nrf2-deficient mice, we show here that Nrf2 also controls the expression of a group of electrophile- and oxidative stress-inducible proteins and activities, which includes heme oxygenase-1, A170, peroxiredoxin MSP23, and cysteine membrane transport (system \( \chi_c \)) activity. The response to electrophilic and reactive oxygen species-producing agents was profoundly impaired in Nrf2-deficient cells. The lack of induction of system \( \chi_c \) activity resulted in the minimum level of intracellular glutathione, and Nrf2-deficient cells were more sensitive to toxic electrophiles. Several stress agents induced the DNA binding activity of Nrf2 in the nucleus without increasing its mRNA level. Thus Nrf2 regulates a wide-ranging metabolic response to oxidative stress.

Oxidative stress conditions or enhanced production of reactive oxygen species (ROS)\(^1\) result from a variety of stimuli including ionizing radiation, exposure to xenobiotics, inflammation, and phagocytosis (1). Treatment of mammalian cells with electrophilic agents usually provokes cellular responses, including transcriptional activation of genes encoding proteins that partake in the defense against oxidative stress. This process is referred to as the electrophile counterattacker response (2). Through analyses of mouse and rat glutathione S-transferase (GST) \( \gamma \) subunit genes and the rat NAD(P)H:quinone oxidoreductase (NQO1) subunit gene, the cis-acting element responsible for the induction by electrophiles was independently identified as an electrophile-responsive element (EpRE) (3) or antioxidant-responsive element (ARE) (4). The consensus ARE sequence has been extensively characterized (5).

The consensus binding sequence of erythroid transcription factor NF-E2 shows high similarity to the ARE/EpRE sequence. Also, the expression profile of Nrf2, one of the NF-E2 subunit factors, overlaps with those of drug-metabolizing enzymes such as GST and NQO1. Based on these facts, we recently demonstrated that transcription factor Nrf2 (6–8) is essential for the coordinated transcriptional activation of genes encoding the drug-metabolizing enzymes, such as GST and NQO1, via AREs/EpREs (9). Nrf2-deficient mice fed with butylated hydroxyanisole, which normally leads to a pronounced up-regulation of Alpha, Pi, and Mu classes of GSTs and NQO1, failed to induce either of these detoxication enzymes in the liver or intestine (9). Since these detoxication enzymes decrease the level of oxidative stress by removing compounds capable of generating ROS or other highly reactive substances, they thereby constitute part of the defense mechanism against oxidative stress (10). Because ARE-type cis-acting sequences are frequently found in the regulatory regions of a number of other oxidative stress-inducible genes (5, 11–13), we hypothesized that Nrf2 might also serve as the key transcription factor activating these genes.

A number of defense proteins and activities in murine peritoneal macrophages are markedly induced upon exposure to electrophilic agents or other oxidative stresses. These proteins include heme oxygenase-1 (HO-1) (14–16), peroxiredoxin MSP23 (17), the cysteine membrane transporter (system \( \chi_c \)) (18) and 60-kDa stress protein A170 (19). HO-1 is prominently induced under various oxidative stress conditions in many different cell types (14). HO-1-deficient embryonic fibroblasts are hypersensitive to the cytotoxicity of both hemin and hydrogen peroxide (15). Induction of system \( \chi_c \) activity increases the intracellular cysteine pool, which consequently augments the synthesis of GSH (20), a potent antioxidant with a short half-life. MSP23 is the murine peroxiredoxin I with antioxidative activity (21). It was recently shown that a mammalian peroxiredoxin isofrom reduces the intracellular hydrogen peroxide level utilizing thioredoxin as an immediate electron donor (22) and protects cells from apoptosis by oxidative stress (23). A170 has a structural domain that interacts with ubiquitin (24) and PKC-\( \xi \) (25). Electrophilic agents, such as diethylmaleate (DEM), and other oxidative stress agents have been reported to induce the proteins HO-1, A170, MSP23, and system \( \chi_c \) activity in peritoneal macrophages (20) and fibroblasts (26). To determine whether these antioxidant stress proteins are also under the regulation of Nrf2, we examined in this study the electrophilic induction of this group of genes in peritoneal macrophages from the nrf2-null mutant mouse.
EXPERIMENTAL PROCEDURES

Culture of Macrophages—Female wild type ICR and nrf2 mutant mice (9) weighing 20–25 g received an intraperitoneal injection of 2 ml of 4% thioglycollate broth. Four days later, macrophages were collected by peritoneal lavage (17). The cells were resuspended at 7.5 × 10⁶ cells/ml and cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum as described previously (17). For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (27) assay, the cells were resuspended at 2 × 10⁵ cells/ml and cultured in medium without fetal bovine serum. After 1 h of culture, stress agents were added to the medium. Final concentrations of the agents in the medium were 100 μM for DEM, paraquat, and hydrogen peroxide (H₂O₂); 80 μM for catechol; 20 milliunits/ml for glucose oxidase (GO); 5 μM for CdCl₂; and menadione (3-butyl-1,4-naphthoquinone); 10 μM for 1-chloro-2,4-dinitrobenzene (CDNB); 20 μM for iodoacetic acid; 2.5 μM for t-butylhydroquinone (t-BHQ) and sodium arsenite (NaAsO₂); and 1 ng/ml for lipopolysaccharide (LPS). The macrophages were harvested at the times indicated in the figure legends. Cell viability was measured by the MTT assay (27) and the trypan blue dye exclusion test.

RNA Blot Hybridization Analysis—Total cellular RNA was extracted from macrophages by RNAzol™ B (TEL-TEST, Inc., Friendswood, TX). The RNA samples (10 μg) were electrophoresed and transferred to Zeta-PROBE GT membranes (Bio-Rad). The membranes were probed with 32P-labeled cDNA probes as indicated in the figure legends. β-Actin cDNA was used as a positive control.

Immunoblotting—Macrophages were solubilized with SDS-sample buffer (without dye or 2-mercaptoethanol), and protein concentrations were estimated by the BCA protein assay (Pierce). The proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and electrotransferred onto Immobilon membrane (Millipore Corp., Bedford, MA). To detect immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit IgG and ECL blotting reagents (Amersham Pharmacia Biotech). Polyclonal rabbit anti-sera raised against rat HO-1, rat MSP23, and recombinant murine A170 were used as described previously (19, 21). Specific antibody was raised against Nrf2 by immunizing rabbits with recombinant Nrf2 protein (amino acids 140–318 fused with an 11× His-tag). An anti-actin antiserum was purchased from Santa Cruz Biotechnology. In competition experiments, a 100-fold excess of unlabeled double-stranded oligonucleotides was used.

RESULTS

Impaired Induction of Antioxidative Stress Proteins in Nrf2-deficient Macrophages—A number of proteins or activities are induced by electrophilic agents in murine peritoneal macrophages. To test whether the electrophilic induction of this group of genes shares a common regulatory mechanism with that of the drug-metabolizing enzymes, we examined their expression in Nrf2-deficient macrophages. Peritoneal macrophages were harvested from nrf2-homozygous and -heterozygous female ICR siblings. The macrophages were then independently challenged with DEM (an electrophilic agent), paraquat (an O₂ generator), GO (an H₂O₂ generator), or CdCl₂ (heavy metal). After challenging macrophages with these agents, we examined expression levels of three oxidative stress-inducible proteins (below) by immunoblotting and RNA blotting analyses. Since wild type and heterozygous mutant mice did not show large differences in induction of the antioxidative proteins, we used both types of macrophages as positive controls.

We first measured the levels of HO-1, MSP23, and A170 proteins 1 h after the start of in vitro culture and found that the basal levels of these proteins in the heterozygous macrophages were similar to those of nrf2-homozygous mutant macrophages (Fig. 1A, lanes 1 and 7). After transfer of the nrf2-heterozygous to in vitro culture, these stress-inducible proteins were gradually induced by unknown mechanisms (compare lanes 1 and 2). The gradual induction of HO-1 and MSP23 expression was not seen in Nrf2-deficient cells (lanes 2 and 8). The important finding here was that whereas all of the stress agents...
tested induced HO-1, MSP23, and A170 in nrf2-heterozygous cells, induction was largely canceled in Nrf2-deficient cells. Closer examination revealed that in nrf2-null mutant cells (lanes 9–12) induction of HO-1 and A170 by DEM and GO was severely affected, but induction by paraquat and CdCl2 was less impaired. In contrast, while MSP23 was markedly induced by these agents in nrf2-heterozygous cells, induction was largely absent in nrf2-null mutant cells.

Quantitative Analysis of Antioxidative Stress Protein Induction by Various Stress Agents—We also examined induction of these proteins by other stress agents: catechol, CDNB, H2O2, iodoacetic acid, sodium arsenite, menadione, and t-BHQ. Quantitative analysis by densitometry of the stained bands is shown in Table I. Menadione and catechol induced HO-1 in the Nrf2-deficient cells at levels comparable with those in nrf2-heterozygous cells, suggesting the involvement of signal-transducing pathway(s) other than the Nrf2 system. Apparently, induction of HO-1 and A170 by DEM and GO was largely abolished. Induction of MSP23 by all of these agents was markedly impaired in nrf2-null mutant cells, while that by t-BHQ and CDNB was largely abolished. Induction of MSP23 mRNA by all of these agents was markedly impaired in nrf2-null mutant cells. The three oxidative stress-inducible genes we addressed here showed roughly comparable variation in the mRNA and protein levels in response to various stress agents (Fig. 1, compare A and B, and data not shown). These results thus indicate that Nrf2 regulates the stress agent-mediated induction of HO-1, MSP23, and A170 gene expression. The results also clearly show that the contribution of Nrf2 to the transcriptional activation of these genes differed based upon the stress-inducing agent.

Induction of the Cystine Transporter x_c^- System Is Defective in Nrf2-deficient Cells—Because oxidative stress agents transcriptionally induce system x_c^- activity in macrophages (20, 26), the stress induction of system x_c^- activity in nrf2-null mutant cells was examined next (Fig. 2A). Whereas nrf2-heterozygous cells show system x_c^- activity comparable with that of wild type cells under both basal and induced conditions (data not shown), the oxidative stress agents DEM, paraquat, GO, and CdCl2 barely induced system x_c^- activity in nrf2-null mutant cells (Fig. 2A). In contrast, LPS, a well known inducer of system x_c^- activity (30), significantly induced the system x_c^- activity even in nrf2-null mutant cells, indicating that LPS induction is mediated through an alternative regulatory pathway rather than the Nrf2 pathway. These results argue that the transcription of the cystine transporter gene may be under the regulatory influence of Nrf2.

It should be noted that system x_c^- activity is necessary to maintain a high GSH level in cultured macrophages, since cysteine is easily oxidized to cystine upon exposure to air (18). While the addition of 500 μM to 1 mM of DEM to the culture medium depletes the intracellular stores of GSH significantly, the addition of 100 μM DEM only diminishes the GSH level minimally, and then its level increases as a result of induced system x_c^- activity (26). As expected, the defect in inducible

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**Table I**

| Agents | HO-1 | MSP23 |
|--------|------|-------|
|         | Nrf2 (+/-) | Nrf2 (-/-) | Nrf2 (+/-) | Nrf2 (-/-) |
| relative absorbance |
| None (1 h) | 0.58 ± 0.02 | 0.50 ± 0.19 | 0.60 ± 0.17 | 0.50 ± 0.25 |
| Name (1 h) | 1.00 | 0.57 ± 0.12 | 0.98 | 0.50 ± 0.16 |
| DEM | 1.67 ± 0.17 | 0.69 ± 0.04 | 1.83 ± 0.04 | 0.54 ± 0.30 |
| CDNB | 2.94 ± 0.21 | 0.77 ± 0.02 | 1.51 ± 0.69 | 0.34 ± 0.08 |
| GO | 1.22 ± 0.02 | 0.57 ± 0.07 | 1.51 ± 0.02 | 0.34 ± 0.21 |
| H2O2 | 1.26 ± 0.10 | 0.55 ± 0.06 | 0.97 ± 0.27 | 0.38 ± 0.14 |
| t-BHQ | 1.91 ± 0.77 | 0.86 ± 0.47 | 1.58 ± 0.07 | 0.44 ± 0.27 |
| Menadione | 6.38 ± 3.95 | 4.54 ± 2.76 | 1.94 ± 0.10 | 0.58 ± 0.21 |
| Catechol | 3.70 ± 0.02 | 1.77 ± 0.06 | 1.75 ± 0.06 | 0.52 ± 0.17 |
| CdCl2 | 1.57 ± 0.14 | 1.21 ± 0.08 | 1.38 ± 0.22 | 0.35 ± 0.15 |
| Paraquat | 1.63 ± 0.03 | 0.88 ± 0.03 | 1.30 ± 0.28 | 0.55 ± 0.25 |
| NaAsO3 | 3.70 ± 1.30 | 1.08 ± 0.51 | 1.01 ± 0.11 | 0.43 ± 0.16 |
| Iodoacetic acid | 2.05 ± 0.10 | 0.98 ± 0.27 | 1.15 ± 0.17 | 0.60 ± 0.27 |
expression of system \( x^- \) activity in \( nrf2 \)-null mutant cells resulted in a decrease in cellular GSH content; after a 24-h incubation with DEM, the GSH level dropped to less than half its original level (Fig. 2B).

Overexpression of \( Nrf2 \) Up-regulates the Activities of the Distal Enhancer of the \( ho-1 \) Gene—The AB1 and SX2 enhancers of the \( ho-1 \) gene (31) contain three and two copies, respectively, of the Maf recognition element (MARE), which largely overlaps with ARE (32). Due to their responsiveness to a wide variety of stress agents including oxidative stress, the MAREs were also named stress-responsive elements (StREs) (31–34). To ask whether \( Nrf2 \) can regulate the expression of this antioxidative stress gene via the \( ho-1 \) AB1 enhancer, we co-transfected a \( Nrf2 \) expression plasmid and a \( ho-1 \) AB1 enhancer-luciferase (p\( \beta \) \( globin \) TATA box-luciferase) reporter (p\( \beta \) \( globin \) TATA box-luciferase) reporter into QT6 fibroblasts (Fig. 3A). DEM (Fig. 3B) and \( Nrf2 \) overexpression (Fig. 3C) both activated \( ho-1 \)-Luc reporter gene expression, with the highest concentrations of these agents generating more than 10-fold activation. This increase was strictly dependent on the presence of the AB1 enhancer (data not shown). We also tested a pRBGP2 reporter that contains three tandem copies of the NF-E2 binding sequence of the \( \beta \) \( globin \) enhancer (7), a sequence that is very similar to the AB1 enhancer sequence. Interestingly, the pRBGP2 reporter responded more efficiently than the p\( \beta \) \( globin \) enhancer-luciferase reporter to DEM and the overexpression of \( Nrf2 \) (Fig. 3B and C). These results indicate that StREs in the AB1 enhancer are actually responsible for the activation of \( ho-1 \)-gene expression and that \( Nrf2 \) can activate the \( ho-1 \)-gene expression through StREs.

Stress Agents Post-transcriptionally Induce DNA Binding Activity of \( Nrf2 \)—Given the clear lack of inductive response in a number of electrophile-inducible genes, it was of interest to determine how oxidative stress agents activate \( Nrf2 \) and thereby induce a group of genes to counteract oxidative stress. To this end, we first examined the level of \( Nrf2 \) mRNA in macrophages under various oxidative stresses and found that the mRNA level was not changed significantly by any of the oxidative stress agents tested (Fig. 4A). In contrast, we found that these same stress agents significantly enhanced the DNA binding activity of \( Nrf2 \) to the StRE of \( ho-1 \) AB1 enhancer.

StRE binding activity in nuclear extracts was strongly induced by DEM treatment of macrophages, as revealed by the retarded band (arrow in Fig. 4B, lanes 1 and 2). The induction of binding activity was not observed in nuclear extracts prepared from \( nrf2 \)-deficient cells (Fig. 4B, lanes 3 and 4). The complex was effectively competed by the addition of an excess of unlabelled StRE, mouse GST \( \gamma \) gene ARE, or chicken \( \beta \) \( globin \) enhancer NF-E2 binding site but not by a yeast Gal4 binding consensus sequence (Fig. 4D). This DNA-protein complex was markedly diminished by treatment with antibodies against mouse \( Nrf2 \) (Fig. 4C, lane 3) and chicken MafK (lane 4), which are partner molecules together comprising the heterodimeric transcription factor complex (34), but the decrease was not obvious using a normal rabbit IgG (lane 5). These results indicate that the DNA-protein complex contains both \( Nrf2 \) and small Maf proteins. The same binding activity was also induced in nuclear extracts of macrophages treated with GO, paraquat, or CdCl2 (Fig. 4E). The \( Nrf2 \) expression level was also examined by immunoblotting analyses of nuclear extracts prepared from the macrophages treated with these stress agents. We found that DEM, GO, paraquat, CdCl2, and CDNB all increased the \( Nrf2 \) level 1.5–3-fold (Fig. 4F). We therefore concluded that post-transcriptional regulation might be involved in the activation of \( Nrf2 \) by electrophilic agents and ROS.

Lack of \( Nrf2 \) Renders Macrophages Sensitive to Oxidative Stress—The analysis thus far clearly indicates that macrophages invoke an electrophile-inducible response upon exposure to oxidative stress agents and that the response is mediated by \( Nrf2 \). To ask whether this response has a major impact on cell viability, we incubated both \( nrf2 \)-null and heterozygous control macrophages with 5–20 \( \mu \)M CDNB for 12 h and measured cell viability by the colorimetric MTT assay. While cell
viability was decreased in both nrf2-null mutant and control macrophages treated with 20 μM CDNB, with 10 μM CDNB, nrf2-null mutant cells were more sensitive to the CDNB treatment than the heterozygous control cells (Table II). Notably, 10 μM CDNB treatment resulted in an approximately 2-fold difference in the MTT assay, and this difference is statistically significant (p < 0.05). The difference in the sensitivity to CDNB between the nrf2-null and -heterozygous cells was much clear when we measured the cell viability by the trypan blue dye exclusion test after 12 h of the CDNB treatment. The viability of the cells was 77 and 16% (mean of two independent experiments) for nrf2-heterozygous and nrf2-null mutant cells, respectively. Nrf2 thus appears to contribute significantly to cellular defense mechanisms against toxic electrophiles.

To highlight the Nrf2 contribution to cellular defense mechanisms, we pretreated macrophages for 36 h with 100 μM DEM, a concentration that potently induces the antioxidative stress response but with low cytotoxicity. The macrophages were subsequently treated with 5 or 10 μM CDNB for an additional 12 h. After incubation, we measured cell viability by trypan blue dye exclusion. The Nrf2-deficient macrophages were more sensitive to treatment with CDNB than the heterozygous control cells. After CDNB treatment, less than 20% of the Nrf2-deficient macrophages were viable (Fig. 5B), whereas more than 95% of the heterozygous cells were viable (Fig. 5A). These results unequivocally demonstrate that there are electrophile-inducible responses mediated by ARE/EpRE and Nrf2 and that the response machinery protects cells against toxic electrophiles and ROS stresses.

**DISCUSSION**

We demonstrate in this study that, in addition to the drug metabolizing enzymes that have already been shown to be regulated by the Nrf2 pathway (9), a group of oxidative stress-inducible genes is also under the immediate transcriptional influence of Nrf2-small Maf heterodimer regulatory proteins. The fact that Nrf2 regulates a group of stress-inducible protein genes via ARE/EpRE, as schematically illustrated in Fig. 6, is intriguing in the context of the physiological origin of these defense mechanisms. In an evolutionary sense, the acquisition of the ARE regulatory mechanism by genes that protect against oxidative stress seems to confer a significant advantage on the survival of living creatures. It should also be noted that,
changing the medium, the cells were incubated with 10 
mutant macrophages were first incubated with DEM for 36 h. After 
stress, which include HO-1, MSP23, and system xc 
tions. Nrf2 is a general regulator of the defense genes against oxidative 
induces transcription of a set of genes that encode antioxidant func-
tviews, see Ref. 32), suggesting that StRE is also competitively bound by various bZip transcription factors. In this regard, we 
previously showed through gene targeting analysis that Nrf2 plays a central role in the regulation of GST and NQO1 gene expression through ARE (9), and the present study has ex-
tended the finding. We found that the inducible expression of the 
ho-1 gene by electrophiles and ROS was severely affected in the 
Nrf2-null mutant macrophages. We also showed that Nrf2 could activate transcription of the reporter gene through the 
AB1 enhancer of the ho-1 gene in a transfection assay. These 
results thus demonstrate that Nrf2 is one of the essential 
regulators of antioxidative stress genes acting through ARE/ 
EpRE and StRE.

should also be noted that Nrf2-dependence of stress-inducible 
gene expression appeared to differ from gene to gene. For instance, none of the electrophilic agents tested induced the 
expression of MSP23 in the nrf2-null mutant macrophages, whereas three of the agents, menadione, CdCl2, and catechol, 
induced ho-1 gene expression to a substantial level even in the 
nrf2-null mutant macrophages. We envisage that this variant 
in Nrf2 dependence reflects differences in the structures of the 
enhancers that mediate the stress signals to gene expression, 
since integration of signals from several stress-sensing pathways should be executed at the level of enhancer sequence. 
Indeed, accumulating evidence suggests that in addition to 
Nrf2, transcription factors NF-κB, AP-1, and heat shock factor 
are also activated in response to various oxidative stresses.

Three lines of evidence further support this hypothesis. First, 
CAAT boxes, metal-responsive elements and NF-κB binding 
sequences are found in the ho-1 enhancers (33, 37). Second, 
menadione is known to effectively activate NF-κB, perhaps by 
generating ROS (38). Third, NF-κB is also known to mediate 
signals from LPS (39). Based on these lines of evidence, we 
speculate that menadione most likely utilized the NF-κB path-
way for ho-1 gene induction in nrf2-null mutant cells. LPS 
induction of system x(m) activity in Nrf2-null mutant cells might 
also be mediated by the NF-κB pathway.

Our present and previous studies uncovered the importance 
of Nrf2 in cellular protection against oxidative stress. This 
thesis was supported by the fact that induction of antioxidative 
stress genes by electrophilic agents was practically absent in 
the Nrf2-deficient macrophages. An important observation 
here is that the activation of Nrf2 by electrophilic agents and 
ROS did not accompany transcriptional induction of the nrf2 
gene (Fig. 4A). Based on this observation, we recently identified 
Keap1, a new factor that binds to the N-terminal Neh2 domain 
of Nrf2 and negatively regulates Nrf2 activity (40). Electrophilic 
agents liberate Nrf2 from Keap1 repression. The results
shown in Fig. 4 suggest a possibility that the stress agents facilitate translocation of Nrf2 from the cytosol to the nucleus. We are now investigating the precise molecular mechanism(s) whereby electrophilic agents and ROS affect interaction between the Neh2 domain of Nrf2 and Keap1.

Elevation of the cellular GSH level is one of the most important events in the electrophile-inducible defense response (2). The increase in GSH has been shown to be achieved by inducible translocation of Nrf2 from the cytosol to the nucleus. Interestingly, hepatic γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step of de novo GSH synthesis, is also induced in mice fed on butylated hydroxyanisole (41) and in HepG2 cells treated with t-BHQ (42). Recently, multiple AREs in the distal enhancer of the ferritin L promoter (12) and metallothionein-I promoter (13) were also shown to be responsive to H2O2. Based on these broad observations, we speculate that many genes that function against oxidative stress are also regulated by Nrf2 via AREs. Thus, the Nrf2-centered gene expression regulatory system creates a coordinated cellular defense against a wide range of electrophilic compounds and ROS. Coordinated and inducible expression of these defense proteins should be important in preventing various free radical-related diseases, such as carcinogenesis, atherosclerosis, ischemia, and neurodegenerative disorders.

Acknowledgments—We thank Dr. Shigeru Taketani for providing polyclonal antibody against rat HO-1 and Drs. Kim-Chew Lim, Kazuhiko Umesono, Priscilla Wilkins Stevens, and Ruth T. Yu for productive discussions and assistance.

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