[18] Transient Enhancement of Heme Oxygenase 1 mRNA Accumulation: A Marker of Oxidative Stress to Eukaryotic Cells

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Introduction

Bacteria respond to oxidative stress by the rapid and transient expression of a large number of genes. Two major regulatory pathways have been recognized to date, namely, the oxyR and soxR systems. The oxyR system regulates nine of the genes induced by hydrogen peroxide in both *Escherichia coli* and *Salmonella typhimurium*.[1] Several of the oxyR-controlled genes (e.g., catalase, glutathione reductase, alkyl hydroperoxide reductase) clearly play a role in defense against oxidative stress. OxyR codes for a regulatory protein which appears to act as an oxygen sensor by being oxidized directly.[2] Another 40 or so genes are turned on by redox cycling agents in *E. coli*, and 9 of these have been shown to have a common regulatory pathway and have been grouped together as the soxR regulon.[3] The detection of expression of the oxyR regulon and/or the soxR regulon should provide an early marker of oxidative stress in prokaryotes.

The binding of several eukaryotic transcription factors such as NF-κB and possibly the fos–jun heterodimer (AP-1) may be redox regulated.[4,5] Expression of the c-*jun* oncogene is stimulated by either UVC radiation (254 nm) or hydrogen peroxide.[6] On the other hand, expression of the human heme oxygenase 1 (HO-1) gene is not induced by UVC radiation, whereas it is strongly induced by oxidizing agents such as UVA (320–380 nm) radiation or hydrogen peroxide.[7] In addition to oxidative stress, the gene is induced by other agents such as phorbol esters, heavy metals, and sodium arsenite.[7] Although this phenomenon was originally observed in fibroblasts cultured from human skin, induction occurs in most human

[1] L. A. Tartaglia, G. Storz, S. B. Farr, and B. N. Ames, *in* "Oxidative Stress: Oxidants and Antioxidants" (H. Sies, ed.), p. 155. Academic Press, London, 1991.
[2] G. Storz, L. A. Tartaglia, and B. N. Ames, *Science* **248,** 189 (1990).
[3] B. Demple and J. D. Levin, *in* "Oxidative Stress: Oxidants and Antioxidants" (H. Sies, ed.), p. 119. Academic Press, London, 1991.
[4] C. Abate, L. Patel, F. J. Rauscher III, and T. Curran, *Science* **249,** 1157 (1990).
[5] R. Schreck, P. Rieber, and P. A. Baeuerle *EMBO J.* **10,** 2247 (1991).
[6] Y. Devary, R. A. Gottlieb, L. F. Lau, and M. Karin, *Mol. Cell. Biol.* **11,** 2804 (1991).
[7] S. M. Keyse and R. M. Tyrrell, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 99 (1989).

cell types and all mammalian cell types so far tested.[8] The induction is clearly related to the cellular redox state since lowering cellular glutathione levels strongly enhances HO-1 mRNA accumulation.[9] For these reasons, altered expression of the HO-1 gene appears to be a fairly sensitive marker of oxidative stress. Indeed, enhanced expression of the HO-1 gene is now being used in several laboratories as a positive control when testing other genes suspected of being oxidant-inducible.

Choice of Assay

Because oxidants induce expression of the HO-1 gene by enhancing the transcription rate,[10] altered expression can be monitored at a variety of levels. Direct measurement of altered transcription rates is probably the most sensitive method, but the run-off transcription assays usually employed for this measurement are more labor-intensive and subject to greater interexperimental variation than assays that measure a later step. One-dimensional sodium dodecyl sulfate (SDS)–poylacrylamide gels are normally sensitive enough to detect induction of *de novo* synthesis of the 32-kDa protein corresponding to HO-1,[11] but background levels are high due to the large number of constitutive proteins in this molecular size range. Induction of HO-1 enzyme activity several hours after the initial treatment is also fairly simple to measure by a spectrophotometric assay,[12] although the biliverdin reductase required for the coupled assay is not available commercially. However, a resolution problem now arises because HO-1 cannot be distinguished enzymatically from the constitutive HO-2 form which is present in various amounts according to cell type.[13,14] The two forms can be distinguished by Western blot analysis, but antibodies to the proteins are not yet commercially available.

With these considerations in mind, the current method of choice is measurement of the accumulation of HO-1 mRNA using a specific cDNA probe. The most commonly used techniques for measuring such accumulation are the dot-blot and Northern blot procedures, the latter being preferred given the high level of nonspecific background that can be associated with the dot-blot procedure. RNA extraction and Northern blot methods

[8] L. A. Applegate, P. Luscher, and R. M. Tyrrell, *Cancer Res.* **51,** 974 (1991).
[9] D. Lautier, P. Luscher, and R. M. Tyrrell, *Carcinogenesis (London)* **13,** 227 (1992).
[10] S. M. Keyse, L. A. Applegate, Y. Tromvoukis, and R. M. Tyrrell, *Mol. Cell. Biol.* **10,** 4967 (1990).
[11] S. M. Keyse and R. M. Tyrrell, *J. Biol. Chem.* **262,** 14821 (1987).
[12] S. Shibahara, T. Yoshida, and G. Kikuchi, *Arch. Biochem. Biophys.* **188,** 243 (1978).
[13] M. D. Maines, G. M. Trakshel, and R. K. Kutty, *J. Biol. Chem.* **261,** 411 (1986).
[14] G. M. Trakshel, R. K. Kutty, and M. D. Maines, *J. Biol. Chem.* **261,** 11131 (1986).

are commonly described in laboratory manuals for molecular biology,[15,16] but we describe here the sequence of procedures that works best for us for our work with HO-1 mRNA. The precise conditions of cell culture and preparation before and after treatment will depend on the cell type employed. We describe the procedure for human fibroblasts cultured as attached monolayers and treated with either UVA radiation or hydrogen peroxide.

Methods

Treatment Procedure

Irradiation with UVA. A broad-spectrum UVA lamp with a large beam area (e.g., we use the UVASUN 3000 lamp supplied by Mutzhas, Munich Germany) is a convenient source of UVA radiation for the processing of many culture dishes simultaneously.

1. Human fibroblast cells (FEK 4[17]) are seeded in 10-cm dishes at a density of 5×10^5 cells in 10 ml Earle's minimal essential medium [supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 0.2% (w/v) sodium bicarbonate, and 15% fetal calf serum (FCS, v/v) (heat-inactivated at 56°)] per dish and cultured for 3 days at 37° in 5% CO_2, at which time they reach 60–80% confluency.

2. Remove the conditioned medium and keep aside at 37°. Rinse the cell monolayers with 10–15 ml phosphate-buffered saline (PBS) at room temperature.

3. Add 5 ml of PBS to each 10-cm dish. The PBS should be supplemented with Ca^{2+}/Mg^{2+} salts (each 0.01% final concentration) just prior to use.

4. Irradiate the culture dishes for the time required at a distance from the UVA source that avoids a temperature rise during irradiation. A fluence range of 0–1 MJ m^{-2} is generally used for the HO-1 gene, and we find that the HO-1 mRNA levels peak at fluences between 400 and 500 kJ m^{-2}. The fluence rate at 30 cm from the UVASUN 3000 lamp source is approximately 300 W m^{-2} as measured by an IL1700 radiometer (International Light Inc., Newburyport, MA).

[15] J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.

[16] L. G. Davis, M. D. Dibner, and J. F. Battey, "Basic Methods in Molecular Biology." Elsevier, New York, 1986.

[17] R. M. Tyrrell and M. Pidoux, *Cancer Res.* **46,** 2665 (1986).

5. After irradiation, aspirate the buffer and rinse the monolayer with 10–15 ml of PBS. Add back the original medium and incubate the cells for 3 hr before extracting total RNA. Addition of fresh medium instead of the conditioned medium induces the gene and gives incorrect estimates of the basal levels of the HO-1 mRNA.

Treatment with Oxidant

1. Cells are grown to 60–80% confluency and prepared for chemical treatment as described above for irradiation with UVA.

2. Add 5 ml of Ca^{2+}/Mg^{2+}-containing PBS to each culture dish and then hydrogen peroxide to a final concentration of 100 μM. A dilute solution of hydrogen peroxide is prepared in sterile water just prior to use. At 240 nm the molar extinction coefficient of hydrogen peroxide is 43.6 M^{-1} cm^{-1}.[18] The appropriate (i.e., nontoxic but effective) concentration of hydrogen perioxide is highly dependent on the cell number and needs to be determined for each given set of experimental conditions.

3. Incubate the cell monolayers with the oxidant for 30 min at 37° in the CO_2 incubator. After chemical treatment, aspirate the buffer containing the oxidant, rinse the monolayers with PBS, and add back the original medium. Incubate cultures for 3 hr at 37° before extracting total RNA.

Isolation of Total Cellular RNA

We use the acid–guanidinum thiocyanate–phenol–chloroform (AGPC) extraction method[19] for isolation of total cellular RNA as it is rapid and a large number of samples can be processed simultaneously. All solutions (except Tris and EDTA) are prepared in water treated with DEPC (diethyl pyrocarbonate).[15] RNase-free glassware and plasticware is used for all manipulations. Use of gloves during all procedures is obligatory for RNA work.

Solutions

Guanidinium thiocyanate stock: 4 *M* guanidinium thiocyanate (250 g plus 293 ml of water), 25 m*M* sodium citrate (pH 7.0) (17.6 ml of 0.75 *M* stock), 0.5% sarkosyl (26.4 ml of 10% stock); guanidinium thiocyanate is dissolved at 65° directly in the manufacturer's bottle; the stock solution is stable at room temperature for 3 months

[18] A. Claiborne, *in* "CRC Handbook of Methods for Oxygen Radical Research" (R. A. Greenwald, ed.), p. 283. CRC Press, Boca Raton, Florida, 1985.

[19] P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162,** 156 (1987).

Solution D: 0.36 ml of 2-mercaptoethanol in 50 ml of guanidinium thiocyanate stock; the solution is stable at room temperature for 1 month
Phenol equilibrated with Tris-Cl (pH 8.0)[15]
Chloroform–isoamyl alcohol mixture (49:1, v/v)
75% Ethanol (v/v)
2 *M* Sodium acetate (pH 4.0)

Procedure

1. Rinse the cell monolayers with 10–15 ml PBS and add 2 ml solution D per 10-cm dish. Cells are lysed directly in the culture dishes for 5 min at room temperature, after which the dishes are left in an inclined position for 2 min to allow the viscous lysate to accumulate on one side of the dish. This allows the collection of 95% of the lysate and is especially useful when a large number of dishes are processed simultaneously.

2. Transfer the lysate to polypropylene tubes with caps and add 0.2 ml of 2 *M* sodium acetate (pH 4.0), 2.5 ml phenol, and 0.4 ml chloroform–isoamyl mixture sequentially. Mix well by rapidly inverting the tube for at least 30 sec after each addition. The mixing steps should be thorough but gentle.

3. Cool suspensions on ice for 15 min and centrifuge at 10,000 *g* for 20 min at 4° to separate the phases.

4. Collect the aqueous phase in a fresh tube and precipitate the RNA with an equal volume of 2-propanol at −20° for at least 1 hr. When a large number of samples are being processed, it may be more convenient to leave the samples overnight at −20° at this step.

5. Pellet the RNA by centrifuging at 10,000 *g* for 30 min and redissolve (room temperature) the pellet in 0.3 ml of solution D. At this step, samples are transferred to 1.5-ml Eppendorf tubes and reprecipitated with an equal volume of 2-propanol (0.3 ml) at −20° for 1 hr.

6. Pellet the RNA by centrifuging in a microcentrifuge at 4°. Wash the RNA pellet twice with 75% ethanol as follows. Add 500 μl of 75% ethanol to each tube and release the RNA pellet by tapping the tube gently against the laboratory bench. Microcentrifuge for 10 min at 4° and then aspirate the supernatant.

7. Vacuum-dry the pellet for 5 min. If RNA is dried for too long, then it does not go into solution easily.

8. Dissolve the RNA in 25 μl of DEPC-treated water by heating to 65° for 10 min followed by quick cooling on ice. Determine the RNA concentration by measuring the absorbance of an aliquot at 260 and 280 nm. The A_{260}/A_{280} ratios obtained should be between 1.95 and 2.0. Store aliquots containing 12–15 μg of total RNA at −20° until further use.

Northern Analysis of HO-1 mRNA

Electrophoresis of RNA. The gel casting trays, combs, and buffer tanks should be used routinely only for RNA work and should be soaked in 1% SDS (w/v) and rinsed well with ultrapure deionized water prior to each use. If RNase is also used in the laboratory, then extra attention should be given to cleaning the gel electrophoresis accessories.[15] We use formaldehyde/agarose gels for separation of RNA.

Solutions

10× MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (final pH between 5.5 and 7.0)*: 0.2 *M* MOPS [3-(*N*-morpholino)propanesulfonic acid], 50 m*M* sodium acetate, 10 m*M* EDTA

Loading buffer (10 ml): 4.8 ml deionized formamide, 1.07 ml of 10× MOPS buffer, 1.73 ml (37%) formaldehyde, 0.533 ml glycerol, 1.033 ml bromphenol blue (saturated solution), 0.834 ml water

Rinse solution*: 75 m*M* NaOH, 100 m*M* NaCl

100 m*M* Tris-Cl (pH 7.5)

Procedure

1. Melt 1.3 g agarose (Bio-Rad, Richmond, CA) in 74 ml of sterile water and add 10 ml of 10× MOPS buffer. Cool to 50° and add 16.2 ml of 37% formaldehyde solution (2.2 *M* final), then pour the contents into a 10 by 15 cm gel casting tray with the comb in position. Formaldehyde gels are cast in a fume hood and allowed to set for 30–45 min. We do not use ethidium bromide in gels which are subjected to Northern transfer.

2. Remove the comb gently and cover the gel with 1× MOPS buffer.

3. To prepare RNA samples for electrophoresis, vacuum-dry aliquots containing 12–15 μg of total RNA to decrease the volume to 5–10 μl. Do not dry completely. Add 20 μl of loading buffer to each sample and heat to 65° for 10 min followed by quick cooling on ice.

4. Load samples and electrophorese at 50 V until the bromphenol blue migrates a distance of approximately 7 cm from the well.

5. After electrophoresis, rinse the gel with deionized water and soak in the rinse solution for 40 min at room temperature on a rocking platform.

6. Neutralize the gel with 100 m*M* Tris-Cl (pH 7.5) for 45–60 min on a rocking platform with one change of buffer and set up the Northern transfer as described below.

* Sterile ultrapure deionized water can be used to make these solutions instead of DEPC-treated water.

It is usually not necessary to electrophorese samples in ethidium bromide-containing gels since RNA prepared by the AGPC method is usually undegraded, provided that proper care has been taken during isolation and electrophoresis. However, if RNA isolation is carried out for the first time, an aliquot of 2 μg of total RNA can be electrophoresed in 1% agarose gels containing ethidium bromide using 0.5× TBE buffer.[15]

Northern Transfer: Capillary Blot Procedure. We use Gene Screen nylon membranes (NEN Du Pont, Dreieich, Germany) for Northern transfer and use the manufacturer's conditions for transfer and hybridization. These are described briefly here.

Solutions

Transfer buffer (pH 6.5)*: 25 m*M* Na_2HPO_4, 25 m*M* NaH_2PO_4

Procedure

1. Place a sponge (small pore, 2 × 15 × 19 cm) in a RNase-free plastic container and add enough transfer buffer to soak the sponge. Do not submerge the sponge in buffer. Air bubbles can be easily removed by poking the sponge with an RNase-free pipette or glass rod.

2. Place a Whatman (Clifton, NJ) paper 3MM (prewet in transfer buffer), cut to a size intermediate between the gel size and the sponge size, on the sponge. Remove entrapped air bubbles by rolling a pipette or glass rod over the paper. Overlay with two more wet pieces of Whatman 3MM paper. Care should be taken to remove air bubbles at each overlay step.

3. Place the gel with the well bottom facing away from the sponge and overlay with a wet piece of Gene Screen membrane cut to the same size as the gel. The nylon membrane should be wetted in transfer buffer for at least 15 min prior to use.

4. Overlay the nylon membrane with one piece of wet and two pieces of dry Whatman 3MM paper cut to the same size as the gel. Place Parafilm strips along the edges of the gel to prevent short-circuiting during transfer.

5. Place a stack of paper towels (8–10 cm high) cut to the same size as the gel and a 1-kg lead weight on top of the stack and leave for at least 16 hr for transfer.

6. Rinse the membrane with transfer buffer to remove residual agarose, place the blot on a Whatman 3MM paper with the transferred RNA side facing upward, and air dry. Bake the blot at 80° for 2–4 hr. After this step the nylon membrane can be stored at room temperature until use.

Hybridization of RNA. We probe the Northern blot for the HO-1 mRNA with the large *Eco*RI fragment (1000 bp) of a full-length cDNA

clone (clone 2/10[7]). The full-length cDNA is inserted into the *Eco*RI sites of the phagemid pBluescript SK (M13$^-$), and we maintain it in the Stratagene (LaJolla, CA) *E. coli* host XL1-Blue {*endA1, hsdR17* (*rk*$^-$, *mk*$^+$), *supE44, thi-1*, λ-, *recA1, gyrA96, relA1, lac*$^-$ [F′, *proAB, lacI*^{q}*Z* Δ M15, Tn*10* (Tet^R)]}. This plasmid DNA (15–20 μg) is digested to completion with *Eco*RI and electrophoresed in a 0.6% low melt agarose gel. The 1000-bp fragment is cut out from the gel and used directly for labeling. This cDNA clone is available from our laboratory. To control for the variation between RNA samples in the same gel, we reprobe each membrane for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA,[20] levels of which are not affected by UVA or other oxidants. Probes for other constitutive mRNA species can also be used as loading controls.

The cDNA probes are labeled with [α-^{32}P]dCTP or [α-^{32}P]dATP using a Random Primed labeling kit supplied by Boehringer Mannheim (Mannheim, Germany). We purify the ^{32}P-labeled fragment on a Elutip-d column (Schleicher and Schuell, Dassel, Germany) according to the procedure recommended by the manufacturer.

Solutions

Prehybridization buffer (recommended for Gene Screen nylon membranes by manufacturer): 50% formamide, v/v (deionized), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA), 0.2% Ficoll, 50 m*M* Tris-HCl (pH 7.5), 1.0 *M* NaCl, 0.1% sodium pyrophosphate, 1.0% SDS (w/v), 10% dextran sulfate (optional), 100 μg/ml denatured salmon sperm DNA

20 × SSC*: 3 *M* sodium chloride, 0.3 *M* sodium citrate

Washing solution 1*: 2× SSC, 0.1% SDS

Washing solution 2*: 0.1× SSC, 0.1% SDS

Procedure

1. For prehybridization, prewet the blot in 6× SSC and place in a plastic bag. Remove as much liquid as possible. Add 12 ml of prehybridization buffer at 42° to the bag, remove air bubbles, and seal the bag. Leave the bag in a 42° water bath with constant agitation for at least 4 hr (can be left for 24 hr). Two filters can be placed in each bag, with the sides containing the RNA facing away from one another.

2. For denaturing the probe, boil in a water bath for 10 min and quick cool on ice for 10 min. Add the denatured probe (specific activity 10^8 cpm/mg) to the prehybridization bag (2–3 × 10^6 cpm/ml, 10–50 ng/ml), remove

[20] P. Fort, L. Marty, M. Piechaczyk, S. E. Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard, *Nucleic Acids Res.* **13**, 1431 (1985).

air bubbles, seal the bag, and leave for hybridization at 42° for 16–24 hr. The probe purified on an Elutip-d column is in a volume of 500 μl, and we add this directly to the prehybridization solution.

3. Collect the hybridization solution and store at −20°. The solution can be reused once; to do so, remove all buffer from the bag after prehybridization and add the hybridization solution which has been boiled in a water bath for 10 min.

4. After hybridization, wash the membrane with constant agitation as follows: (a) once in 100 ml of 2× SSC for 5 min at room temperature; (b) once in 100 ml of washing solution 1 for 30 min at 50°; and (c) once in 100 ml of washing solution 2 for 30 min at 65°. For the HO-1 cDNA probe, the third washing step (c) is usually not necessary.

5. Air dry the membrane, wrap it in Saran wrap and expose to a preflashed film at −70°.[15] Because the membranes are usually rehybridized with the GAPDH probe, they should be left slightly damp.

6. Prior to rehybridization, the labeled probe is stripped from the membrane by boiling in washing solution 2 for 40 min with one solution change. It is recommended that the labeled probe be stripped off from the membrane soon after autoradiography if rehybridization is to be carried out. The stripped Northern blot can be air dried and stored between sheets of Whatman 3MM paper at room temperature.

A typical Northern blot analysis of total cellular RNA isolated by the AGPC method and probed for the HO-1 and GAPDH mRNA species is shown in Fig. 1. For this experiment cell cultures were exposed to a range of fluences of UVA radiation in the presence of 5 m*M* *N*-acetylcysteine.

Quantification of Northern Analysis. Because there is a fluence-dependent change in HO-1 mRNA levels, it is necessary to carry out autoradiography for different periods of time. Autoradiographs in which the control sample (see Fig. 1) which has the lowest signal intensity is clearly visible and the band with the highest signal intensity is still in the linear range

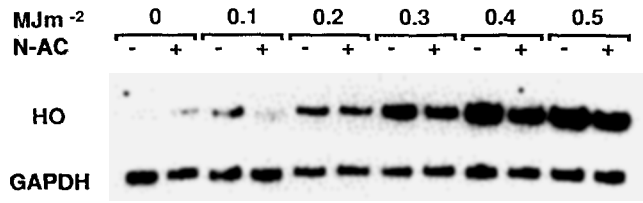

FIG. 1. Human skin fibroblasts (FEK 4) were irradiated with increasing fluences of UVA in the presence (+) or absence (−) of 5 m*M* *N*-acetylcysteine (N-AC). Total RNA was subjected to Northern blotting and probed first for HO-1 mRNA and then for GAPDH mRNA.

should be used for densitometric analysis. A detailed densitometric analysis of autoradiographs with different exposure times needs to be done for one experiment, after which it is possible to judge the signal intensity visually.

Radioactive signals are quantified in our laboratory by densitometry using an Elscript 400 (Hirschmann) densitometer with evaluation software. We usually scan vertically down each track rather than scanning the band of interest horizontally along the gel. The area under the curve of the band of interest is integrated with the evaluation software supplied with the densitometer. Areas determined by densitometry of autoradiographs in Fig. 1 are shown in Table I.

Calculation of Increase in HO mRNA

The HO-1 mRNA signal intensity increases severalfold over basal levels in response to oxidative stress, and this response can be modulated by chemical agents such as *N*-acetylcysteine (see Fig. 1). The first step in the quantification is to normalize for the variation in loading between samples on the same gel using the GAPDH mRNA signal (or any other constitutive mRNA) on autoradiographs. A GAPDH ratio is calculated as follows:

1. Calculate the mean areas under the curves of all the GAPDH bands on a gel from the autoradiograph.

TABLE I
AREAS DETERMINED BY DENSITOMETRY FOR HO-1 mRNA AND GAPDH mRNA

Lane number[a]	HO-1 area	GAPDH area	GAPDH ratio	GAPDH ratio × HO-1 area
1	3.21	33.89	0.87	2.79
2	8.01	26.42	1.12	8.97
3	8.86	23.82	1.24	10.99
4	6.19	30.63	0.97	6.00
5	15.57	18.03	1.64	25.53
6	13.23	19.41	1.53	20.24
7	53.22	20.52	1.44	76.64
8	30.13	23.51	1.26	37.96
9	109.17	48.72	0.61	66.59
10	55.04	33.52	0.88	48.44
11	93.16	33.65	0.88	81.98
12	73.72	43.21	0.69	50.87

[a] See Fig. 1; lanes are numbered from left to right.

2. Divide the mean by the GAPDH area of each sample to obtain a GAPDH ratio for each lane. If equal amounts of total RNA were loaded in all tracks, then the signal intensity would be the same in all the tracks. This is usually not the case and thus the need for this normalization step.

3. Multiply the area under the curve of each HO-1 band by the corresponding GAPDH ratio. The numbers thus obtained are normalized for the variation between samples on the same gel.

4. The calculated area of the control sample (Table I, lane 1) is used as a unit to calculate the increase(-fold) in HO-1 mRNA above basal levels. A plot of relative increase in HO-1 mRNA levels as a function of fluence is shown in Fig. 2A.

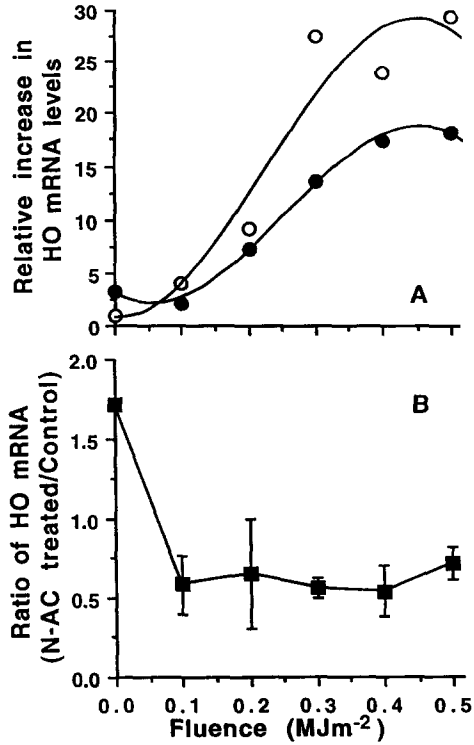

FIG. 2. The HO-1 and GAPDH mRNA signals were quantified by densitometry. The GAPDH signal was used as an internal control to normalize for the variation in loading between samples. (A) Normalized HO-1 mRNA signals are expressed as the relative increase above basal levels and plotted as a function of fluence. (○) Control cells, (●) cells treated with *N*-acetylcysteine. (B) The ratios of the normalized HO-1 mRNA signal areas in samples treated with 5 m*M* *N*-acetylcysteine (N-AC) and control samples are plotted as a function of fluence.

The increase(-fold) of HO-1 mRNA varies between experiments and thus error bars are large; however, the pattern is consistent for any agent. To quantify the effect of a test agent it is useful to calculate the ratios of the HO-1 mRNA areas (normalized) in untreated and treated samples of at least three experiments. Such ratios with their standard deviations are plotted against fluence in Fig. 2B.

Transient enhancement of HO-1 mRNA accumulation appears to be an extremely sensitive marker of oxidative stress, and it has been employed in assays in several laboratories. In this chapter we have described detailed methodology based on the Northern blot procedure for the comparative measurement of HO-1 mRNA levels and provide an example in which increased accumulation of HO-1 mRNA after a mild oxidative stress (UVA radiation) is suppressed by a free radical scavenging antioxidant (*N*-acetylcysteine).

Acknowledgments

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[19] Assays for Regulation of Gap Junctional Communication and Connexin Expression by Carotenoids

By JOHN S. BERTRAM and LI-XIN ZHANG

Introduction

Compelling epidemiologic evidence has shown that certain carotenoids have cancer chemopreventive activities.[1] However, their mode of action in this respect is unclear. Carotenoids as a group are considered to possess antioxidant properties,[2] and a limited number act as provitamin A sources in mammals.[3] In view of the postulated role of oxidative damage in carcinogenesis[4,5] and the known activity of retinoids as cancer chemopreventives,[6]

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[2] N. I. Krinsky, *Free Radical Biol. Med.* **7,** 617 (1989).
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