Cytoplasmic HuR Expression Correlates with Poor Outcome and with 
Cyclooxygenase 2 Expression in Serous Ovarian Carcinoma

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Abstract

Cyclooxygenase-2 (COX-2) expression has been shown to associate with poor prognosis in ovarian cancer, and an mRNA stability protein HuR has been shown to enhance expression of COX-2 in tissue culture conditions. We found cytoplasmic immunoreactivity for HuR protein in 52% (233 of 445) of serous-type ovarian carcinoma specimens, and it associated with high COX-2 expression (P = 0.0045) and with clinicopathological variables, including poor prognosis (P < 0.0001) and high grade (P < 0.0001). In ovarian cancer cells in vitro, a small interfering RNA against HuR and leptomycin B, an inhibitor of nucleocytoplasmic translocation of HuR, inhibited COX-2 expression. Our results show that cytoplasmic HuR expression associates with poor outcome in ovarian cancer, and one plausible explanation for this finding may be related to the ability of HuR to induce COX-2 expression.

Introduction

Regulation of mRNA stability is an important component of eukaryotic gene expression (1, 2). This is especially evident for short-lived transcripts, including those of certain proto-oncogenes, cytokines, and cytokine-response genes. Because such genes are usually transcribed in a rapid but transient manner, this combination of regulatory steps of gene expression facilitates tight control over these potentially harmful proinflammatory and transforming factors. One of the best-characterized cis-elements in the regulation of mRNA turnover are AREs located in the 3′ untranslated region of many unstable messages (1). Several ARE-binding proteins have been identified, of which HuR (or HuA) is a Hu family member of RNA-binding proteins that are related to Drosophila embryonic lethal abnormal vision protein (2, 3). HuR is ubiquitously expressed, whereas the other family members (HuB/Hel-N1, HuC, and HuD) are neuron-specific (3). Intracellular localization of HuR is predominantly nuclear, but it is thought to shuttle between nucleus and cytoplasm, which suggests that HuR binds to ARE-containing mRNAs in the nucleus, and this complex is then transported to the cytoplasm (2). The use of cell-permeable peptides has identified two distinct nucleocytoplasmic export mechanisms for c-fos mRNA (4). One of these export proteins is export receptor CRM1, which binds to leucine-rich nuclear export signals that are present in two protein ligands of HuR, pp32, and APRIL. Shuttling of pp32 and APRIL is totally blocked by inhibition of CRM1 with a fungal metabolite named LMB. Although LMB was shown to cause toxic side effects in Phase I trials for treatment of cancer, it is an important research tool due to its high specificity to CRM1 (5).

COX-2 mRNA contains HuR-binding AREs in its 3′ untranslated region (6–10). Consistent with its function as an mRNA stability protein (2), overexpression of HuR increased COX-2 expression in colorectal cancer cells (7), and down-regulation of HuR inhibited COX-2 expression in breast cancer cells (8). IL-1α was the first agent that was shown to increase the half-life of COX-2 transcript (11), and IL-1β was shown recently to increase cytosolic content of HuR protein/COX-2 mRNA complexes (10). Furthermore, expression of COX-2 is elevated in several human malignancies (12), and it has been shown to be an independent prognostic factor in adenocarcinoma of the esophagus (13) and the ovary (14). Because it is not known whether expression of ARE-binding proteins, including HuR, associate with prognosis or with COX-2 expression in human carcinomas, we have correlated HuR expression with clinicopathological parameters in serous ovarian carcinoma specimens, and studied the effect of HuR siRNA and LMB on COX-2 expression in ovarian cancer cells.

Materials and Methods

Patients. The study consisted of 474 consecutive patients treated for serous ovarian carcinoma at the Department of Obstetrics and Gynecology of the Helsinki University Central Hospital between the years 1964 and 2000, whenever a histological specimen was available in the pathology files (15). The study was approved by the local Ethics Committee. The histology was determined by a gynecological pathologist and verified by another pathologist (R.B.). The clinical information was obtained from the patient records and survival data from the Population Register Center. The median age of the patients was 57 years (range 15–88 years) at the time of diagnosis. The median follow-up time of patients alive at the end of study period was 5.2 years (range, 0.4–36.1 years). The patients were operated on by gynecological oncologists, and the clinical staging and grading was performed according to Federation of International Gynecology and Obstetrics. The tumor samples for the study were obtained at primary surgery before any chemotherapy. Patients with recurrent disease obtained chemotherapy and/or underwent secondary surgery when clinically meaningful.

Tissue Microarray Analysis. Tissue microarrays were constructed from formalin-fixed and paraffin-embedded archive specimens as described previously (16). In brief, a representative tumor area was selected from H&E-stained sections of each tumor by our pathologist (R.B.). Four tissue cores (diameter 0.8 mm) from different parts of the specimen were obtained from each tumor block and replaced into recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). The purpose for staining of several cores from a single tumor block was to reduce the possible effect of intraspecimen heterogeneity in antigen expression. The samples were then cut with a microtome into 5-μm sections, and the presence of cancer cells was verified on H&E-stained sections.
**Immunohistochemistry.** Specimens were deparaffinized and antigen retrieved using microwave oven [4 × 5 min in 700 W in 0.01 M Na-citrate buffer (pH 6.0)]. The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and in blocking solution (1:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining for HuR was performed with a monoclonal antibody 19F12 (17), which was raised against a unique peptide from the NH2 terminus of HuR, in a dilution of 1:10,000 (1.0 μg/ml) in PBS containing 0.1% sodium azide and 0.5% BSA. The specimens were incubated with the antibody at room temperature overnight. The sections were then treated with biotinylated horse antimouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA) and avidin-biotin peroxidase complex (Vectastain ABCComplex; Vector Laboratories). The peroxidase staining was visualized with 3-aminon-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO), and the sections were counterstained with Mayer's hematoxylin. To confirm the specificity of the staining nine histological sections with serous ovarian carcinoma were stained with and without the antigenic peptide for 19F12 (10 μg/ml) for 1 h at room temperature before the staining procedure. Immunostaining protocol for COX-2 has been described previously (13, 18), and the results on the COX-2 staining in ovarian cancer specimens will be described elsewhere (19).

**Scoring.** Only those tumors (445 of 474; 96%) from which at least three cores remained analyzable after the immunostaining procedure were scored. The intensity of staining was scored separately in cancer cells and in nonneoplastic stromal cells by two independent and blinded investigators (T-L. E. and A. S.). In addition, intensity of nuclear positivity versus cytoplasmic positivity was scored separately in cancer cells and in nonneoplastic stromal cells, i.e., nuclear intensity > cytoplasmic intensity and *vice versa.* Scoring of COX-2 has been described previously (18, 19), and scores 0 and 1 were considered as low category, and scores 2 and 3 as high category. In both cases (i.e., HuR and COX-2) every dot was scored by the two independent investigators, and the score of the tumor was the most common score found.

**Statistical Analysis.** The correlation between HuR staining and clinically relevant and prognostic variables was assessed by using the χ2 test. Probability of survival was estimated using the Kaplan-Meier method. Survival curves and probabilities of 5-year survival were compared with the log-rank test. Disease-specific survival time was defined as the time from primary surgery to death of the patient from ovarian cancer or to the end of the follow-up. Of the 445 patients with known HuR score, 288 died during the follow-up. Of these cases, 250 (87%) died from ovarian cancer, 6 (2%) from another cancer, and 32 (11%) from another disease. The 38 deaths due to causes other than ovarian cancer and without any sign of dissemination of the ovarian cancer were treated as censored cases, as well as those patients who were alive at the end of the follow-up (n = 157). Multivariate survival analyses were performed using the Cox proportional hazards model entering the following variables: HuR score (cytoplasmic negative versus positive; score of the tumor was the most common score found), stage (1, 2, and 3), and residual tumor size (≤1 cm versus >1 cm). All of these data were available from 374 patients. Tumor size and COX-2 expression are not independent prognostic variables in this material (19).

**Cell Culture Experiments.** Human ovarian adenocarcinoma cell line OVCAR-3 was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM supplemented with 10% FCS (Life Technologies, Inc., Invitrogen, Paisley, Scotland), 2 mM l-glutamine, 0.1 units streptomycin, and 0.1 units penicillin (Bio Whittaker Europe, Verviers, Belgium). The siRNA duplexes against HuR were synthesized by Dharmaco Inc. (Lafayette, CO), and the sequences were published recently (8). The β-actin siRNA was also from Dharmaco Inc. The day before transfection, OVCAR-3 cells were trypsinized and plated in OptiMEM medium (Life Technologies, Inc., Invitrogen) with 10% antibiotic FCS without antibiotics in a split ratio of 1:2 into 12-well plates. Transient transfection of siRNA molecules was carried out by using Oligofectamine Reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) following the manufacturer’s instructions. First OptiMEM medium (8 μl) and Oligofectamine (2 μl) were mixed, and at the same time OptiMEM (85 μl) was mixed with the HuR or β-actin siRNA duplexes (5 μl). These two reactions were incubated separately for 10 min at room temperature, after which they were combined and incubated for 20 min at room temperature for complex formation. The cells were washed once with serum-free and antibiotics-free OptiMEM, and then 400 μl of OptiMEM and 100 μl of the transfection mixture were added onto the cells. The cells were then incubated for 4 h in 37°C at 5% CO2 in a cell incubator, after which 250 μl of 3x complete culture medium was added. The cells were incubated with the siRNA molecules (200 nm) for 72 h, and IL-1β (10 ng/ml; R&D Systems, Minneapolis, MN) was added for the last 24 h of the incubation period. In the LMB (Sigma Chemical Co.) experiment the cells were incubated with LMB (5–10 ng/ml) in combination with IL-1β for 24 h.

**Western Blot Analysis.** The cells were lysed in 400 μl (95°C) lysis buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, and 0.002% bromphenol blue], after which the samples were heated for 4 min in 95°C and centrifuged at 14,000 × g for 10 min. Thereafter 50 μl of the supernatant was analyzed by using 12% SDS-PAGE. Alternatively the cells were lysed in 1 ml of radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris (pH 8.0)] supplemented with Complete mini protease inhibitor mixture tablet (Boehringer, Mannheim, Germany) and centrifuged at 14,000 × g for 15 min. Protein concentration was measured from the supernatant with BCA Protein Assay Kit (Pierce, Rockford, IL), and samples containing 75 μg of total protein were resuspended in sample loading buffer [74 mM Tris-HCl (pH 6.8), 2% SDS, 12% glycerol, 5% β-mercaptoethanol, and 0.015% bromphenol blue] and separated by 12% SDS-PAGE. The proteins were transferred electrophoretically to Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom). Nonspecific binding was blocked by TBS-NP40 and 5% low-fat dry milk solution overnight at 4°C. For immunodetection, the membranes were incubated with the 19F12 monoclonal HuR antibody (1:100,000) or with mouse antihuman monoclonal COX-2 antibody (160112; 1:1000 dilution, Cayman Chemical Co., Ann Arbor, MI). The membranes were then washed three times in TBS-NP40 and incubated with the sheep anti-mouse antibodies conjugated to horseradish peroxidase (1:2,000 dilution). Loading was controlled by goat antihuman β-actin antibodies (1:500 dilution; Santa Cruz Biotechnology) with donkey antigoat antibodies conjugated to horseradish peroxidase as the secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology). After four washes with TBS-NP40, the signals were visualized by enhanced chemiluminescence with Super Signal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer’s protocol and by using Multilmage FC Light Cabinet (Promega Co., Madison, WI) and the digital imaging FluorChem 8800 software (Alpha Innotech Co., San Leandro, CA).

**Results and Discussion.**

**HuR Protein Expression in Serous Ovarian Carcinoma Specimens.** Nuclear immunoreactivity for HuR was observed in all but 2 of the 445 serous-type ovarian carcinoma specimens, and cytoplasm of the cancer cells was positive in 52% (233 of 445) of the cases. In the “cytoplasm positive category” nuclear staining was more intense than cytoplasmic one in 86% (200 of 233) of the samples and *vice versa* in 14% (33 of 233). Although heterogeneous nuclear HuR positivity was observed in stromal cells, cytoplasm of these nonneoplastic cells remained negative (Fig. 1, A and B). Specificity of the 19F12 antibody was confirmed by using the antigenic peptide as a blocking reagent. This preadsorption procedure completely abolished all immunopositivity, as shown in the insets of Fig. 1, A and B. The percentage agreement between the two independent and blinded investigators in allocation of the tumors into cytoplasm-negative and cytoplasm-positive categories was 96%. All of the specimens with discordant scores (n = 18) were reevaluated by the two investigators using a multichip probe, and the consensus score was used for additional analyses.

HuR expression has been investigated previously in brain tumors and in colorectal cancer (6, 7). In brain tumors, expression of HuR mRNA was detected regardless of their origin or degree of malignancy (6). However, immunoreactivity for HuR was expressed predominantly in high-grade brain tumors (glioblastoma multiforme 8 of 8 and medulloblastoma 1 of 1), but only in 1 of 9 meningiomas and in 0 of the 3 astrocytomas. Staining pattern was predominantly nuclear with lesser cytoplasmic positivity. Similarly, elevated immunoreactivity of HuR has been detected in colorectal cancer when compared...
Survival as analyzed by Kaplan-Meier method (P < 0.0001, log-rank test). Eight nuclei are circled by a broken line, and the original magnification was ×400. B, in this ovarian carcinoma specimen HuR immunoreactivity was present in both the nucleus and the cytoplasm of the cancer cells. Fig. 1. HuR protein is expressed in the cytoplasm of a subset of serous-type ovarian carcinoma specimens, which correlates with poor outcome. A, an ovarian carcinoma specimen, in which HuR immunopositivity was restricted to the nucleus of the cancer cells. Adsorption with an antigenic peptide totally blocked the signal (inset). Eight nuclei are circled by a broken line, and the original magnification was ×400. B, in this ovarian carcinoma specimen HuR immunoreactivity was present in both the nucleus and the cytoplasm of the cancer cells. Antigen adsorption totally blocked both nuclear and cytoplasmic signals obtained by the antibody (inset). Nuclei are circled and the original magnification was as described for A. C, expression of HuR was detected in the cytoplasm of the cancer cells in 52% (233 of 445) of serous ovarian carcinoma specimens, and it correlated with reduced disease-specific survival as analyzed by Kaplan-Meier method (P < 0.0001, log-rank test).

Table 1 Association of cytoplasmic HuR immunopositivity with clinicopathological parameters in serous-type ovarian carcinoma

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>n total</th>
<th>HuR positive(n %)</th>
<th>P* according to HuR positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>445</td>
<td>233/445 (52%)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Age at diagnosis ≤57</td>
<td>445</td>
<td>110/225 (49%)</td>
<td>NS (P = 0.1382)</td>
</tr>
<tr>
<td>&gt;57</td>
<td>132/220 (56%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>437</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1</td>
<td>63/168 (38%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>65/115 (57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>101/154 (66%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>442</td>
<td></td>
<td>NS (P = 0.0589)</td>
</tr>
<tr>
<td>1</td>
<td>40/92 (43%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36/63 (57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>124/239 (52%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>32/48 (67%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>439</td>
<td></td>
<td>NS (P = 0.8855)</td>
</tr>
<tr>
<td>≤10 cm</td>
<td>73/138 (53%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 cm</td>
<td>157/301 (52%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual tumor size ≤1 cm</td>
<td>91/194 (47%)</td>
<td></td>
<td>0.0028</td>
</tr>
<tr>
<td>&gt;1 cm</td>
<td>125/202 (62%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>426</td>
<td></td>
<td>0.0045</td>
</tr>
<tr>
<td>Low</td>
<td>52/126 (41%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>169/300 (56%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a 2 test.
* b Not significant.

HuR in Ovarian Cancer

with adjacent nonneoplastic crypt epithelium (7). In serous ovarian carcinomas we found homogeneous nuclear positivity in practically all of the tumors in the cancer cell compartment, but cytoplasmic positivity was observed only in a subset of the tumors. Because nucleocytoplasmic translocation of HuR is necessary for its activity (2, 4, 8, 10), and cytoplasmic localization of HuR has been correlated with chemically induced lung hyperplasias and neoplasias of the lung in mice (20), we rationalized that cancer cell cytoplasmic HuR expression could be a useful marker in clinical cancer specimens.

Association of HuR with Survival. Cytoplasmic HuR immunoreactivity associated with decreased disease-specific survival (P < 0.0001) of the 445 serous ovarian carcinoma patients (Fig. 1C). Five-year disease-specific survival in cytoplasm-negative category was 60% (95% CI, 53–67) and in cytoplasm-positive category 39% (95% CI, 32–46). However, there was no statistically significant difference, when the category “nuclear staining more intense than cytoplasmic” (5-year survival 38%; 95% CI, 30–45) was compared with category “cytoplasm staining more intense than nuclear” (5-year survival 43%; 95% CI, 25–62). Thus, cytoplasmic immunopositivity for HuR predicted poor outcome in serous ovarian carcinoma patients, but the two categories of cytoplasmic immunopositivity (cytoplasm > nuclear versus nuclear > cytoplasm) did not differ with regard to survival.

Association of HuR with Clinicopathological Parameters. Cytoplasmic HuR expression was significantly more frequent in high-grade tumors (P < 0.0001) and in cases with residual tumor size >1 cm (P = 0.0028; Table 1). No significant association was found between cytoplasmic HuR and age at diagnosis, stage, or tumor size. However, there was a trend of correlation with advanced stage (P = 0.0589). Our data (19) and recent work reported by Denkert et al. (14) indicate that high COX-2 expression correlates with poor outcome of patients with ovarian cancer. Because cytoplasmic localization of HuR has been linked to induction of COX-2 expression (6–10), we investigated correlation of cytoplasmic HuR staining with COX-2 expression. Indeed, cytoplasmic HuR expression correlated with high expression of COX-2 protein in the ovarian cancer specimens (Table 1). In fact, frequency of COX-2-expressing tumors was progressively increased, when the category “cytoplasm negative” was compared with the category “nuclear staining more intense than cytoplasmic” and to the category “cytoplasmic staining more intense than nuclear” (Table 2). COX-2 may, thus, be one of the signals of cytoplasmic expression of HuR in cancer cells. Because COX-2-derived prostanoids can induce solid tumor growth, invasion, and metastasis (12), it may well be that part of the tumor-promoting effect of HuR is dependent on induction of COX-2 expression.

Multivariate Analysis. Multivariate survival analysis was performed to evaluate the independence of cytoplasmic HuR expression as a prognostic factor. We have identified previously that age >57, high grade, advanced stage, and residual tumor size >1 cm are independent prognostic variables in this ovarian cancer material, whereas tumor size >10 cm and high COX-2 expression were not (19). Inclusion of cytoplasmic HuR expression did not add significant independent prognostic information, when the above-mentioned independent covariates were included to the analysis.

Effect of HuR siRNA and LMB on COX-2 Expression in Ovarian Cancer Cells. To study the effect of HuR expression on ARE-containing mRNA molecules in ovarian cancer cells, we chose to use HuR-targeted siRNA molecules to inhibit HuR expression and LMB, which inhibits transport of HuR-binding proteins, pp32 and APRIL.

Table 2 Association of cytoplasmic HuR immunopositivity with elevated COX-2 expression in serous-type ovarian carcinoma specimens (n = 426)

<table>
<thead>
<tr>
<th>HuR immunopositivity</th>
<th>COX-2 positive(n %)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm negative</td>
<td>131/205 (64%)</td>
<td>0.0144</td>
</tr>
<tr>
<td>Nuclear &gt; cytoplasm</td>
<td>143/189 (76%)</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm &gt; nuclear</td>
<td>26/32 (81%)</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm positive</td>
<td>169/221 (77%)</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

* a 2 test.
* b Cytoplasm positive compared to cytoplasm negative.
via CRM1 from nucleus to the cytoplasm (2, 4, 5). IL-1β induced COX-2 expression in OCVAR-3 cells (Fig. 2), as was reported recently by Denkert et al. (14). When OCVAR-3 cells were transfected with HuR siRNA (200 nm), expression of HuR and IL-1β-induced COX-2 expression were reduced (Fig. 2A), β-Actin siRNA served as a control, and it did not reduce expression of either HuR or COX-2.

IL-1α has been shown previously to increase the half-life of COX-2 mRNA in immortalized ECV cells (11), and IL-1β induced translocation of HuR bound to COX-2 mRNA from nucleus to the cytoplasm in rat mesangial cells (10). Our present data show that HuR plays an important role in IL-1β-induced expression of COX-2 also in transformed cells. Furthermore, it was shown recently that LMB inhibits COX-2 expression in monocytes, and in breast and colon cancer cells (21). One likely explanation for this observation is that LMB inhibits CRM1-mediated nucleocytoplasmic transport of HuR protein/COX-2 mRNA complexes. As shown in Fig. 2B, LMB inhibited IL-1β-induced COX-2 expression in OCVAR-3 cells in a concentration-dependent manner. All of this indicates that HuR is necessary for COX-2 expression in ovarian cancer cells.

Our results show that cytoplasmic HuR expression associates with poor outcome in patients with serous ovarian carcinoma. Thus, HuR is the first mRNA stability protein, expression of which has been linked to reduced survival in carcinoma patients. Because expression of COX-2 correlated with cytoplasmic HuR expression, and overexpression of COX-2 has been shown to be a marker for reduced survival in ovarian cancer patients, one plausible explanation for this phenomenon is that HuR mediates its effect, at least in part, by induction of COX-2 expression. This point is additionally supported by our cause-and-effect data that indicate that inhibition of HuR reduced COX-2 expression in ovarian cancer cells.

Fig. 2. Effect of HuR siRNA and LMB on expression of COX-2 protein in ovarian adenocarcinoma cells. A. OCVAR-3 cells were treated with siRNAs specific for HuR or β-actin (200 nm) for 72 h, and IL-1β (10 ng/ml) was added for the last 24 h of the incubation period. Proteins for COX-2, HuR, and β-Actin (as a loading control) were detected by Western blot analysis. B, the cells were treated with LMB (5–10 ng/ml) and IL-1β (10 ng/ml) for 24 h after which COX-2 and β-actin were detected by Western blot analysis.

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References

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