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Induction of heme oxygenase-1 in normal and malignant B lymphocytes by 15-deoxy- $\Delta^{12, 14}$ -prostaglandin J₂ requires Nrf2

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Abstract

Heme-oxygenase-1 (HO-1) is induced in response to oxidative stress and is believed to be a cytoprotective and anti-inflammatory enzyme. It is unknown whether normal or malignant human B lineage cells express HO-1. 15-deoxy- $\Delta^{12, 14}$ -prostaglandin J₂ (15d-PGJ₂) is an interesting electrophilic lipid mediator able to increase oxidative stress in B cells. Here, we tested normal and malignant human B lineage cells for their ability to express HO-1 in response to 15d-PGJ₂, as well as the signaling pathways required for HO-1 expression. 15d-PGJ₂ potently induced HO-1 protein expression in normal and malignant B cells. Malignant B cells exhibited a greater induction of HO-1 protein compared to normal B lymphocytes. Using siRNA directed against the transcription factor Nrf2 and B cells isolated from Nrf2-deficient mice, we show that HO-1 induction by 15d-PGJ₂ is dependent on Nrf2. These results show that, compared to normal B lymphocytes, malignant B cells have a greater capacity to increase their HO-1 protein levels in response to 15d-PGJ₂. We speculate that the ability to highly express HO-1 by malignant B cells could confer a survival advantage.

Keywords

heme oxygenase-1; 15d-PGJ₂; Nrf2; B lymphocytes; B cell malignancy

Introduction

Heme oxygenases (HO) are rate-limiting enzymes that convert heme to equimolar concentrations of biliverdin, carbon monoxide (CO) and iron (Fe²⁺); biliverdin is subsequently converted to bilirubin by biliverdin reductase (BVR) [1]. There are two well-characterized HO isoforms in humans: HO-1 and HO-2. HO-2 is constitutive, whereas the expression of HO-1 is inducible. HO-1 expression is elevated in certain cancers, including liver [2], kidney [3], and prostate [4]. In this regard, HO-1 can be viewed as pro-tumorigenic [5]. HO-1 may also play an important role in immune system development and function. Recently, HO-1 expression was described in T regulatory cells, where HO-1 activity mediated some of the suppressive effects of these cells [6]. HO-1 also inhibits T cell adhesion and expression of HLA-DR on the surface of IFN- γ primed endothelial cells [7]. Interestingly, HO-1-deficient mice have fewer B220⁺ cells within the lymph nodes and exhibit abnormal immunoglobulin profiles [8], suggesting that HO-1 plays an important role in B cell function. However, the expression and function of HO-1 in human B cells or key B cell malignancies are unknown.

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of diseases characterized by uncontrolled malignant B-lineage cell proliferation [9]. NHL is the fifth most frequently diagnosed cancer in the United States [10] and is the leading cause of cancer death in individuals between the ages of 20-40 years [11]. Mantle cell lymphoma is a subtype of B-cell NHL and carries a distinct immunologic and genetic phenotype [12]. Multiple myeloma (MM) accounts for 10% of all hematological malignancies and is characterized by uncontrolled proliferation of plasma cells [13]. Despite advances in treatment options, both MM and mantle cell lymphoma are aggressive hematological malignancies with poor survival outcomes (i.e. less than 5 years) [13, 14], due, in part, to chemotherapy resistant tumor cells [15]. This resistance may be related to the selective expression of key proteins that permit tumor cells to survive.

Prostaglandins are small lipid mediators synthesized from arachidonic acid via the activity of cyclooxygenases (Cox-1 and Cox-2). The first prostaglandin produced in the pathway is PGH₂ which is metabolized by tissue-specific isomerases to prostanoids (PGD₂, PGE₂, PGF_{2α}, and PGI₂) and thromboxanes. 15d-PGJ₂, forms spontaneously from the dehydration of PGD₂ [16] and has several biological actions many of which relate to its ability to generate reactive oxygen species (ROS) [17]. 15d-PGJ₂ has been reported to increase HO-1 expression in cells such as fibroblasts and macrophages [18-20]. The transcriptional up-regulation of HO-1 by 15d-PGJ₂ has been reported to be mediated by several transcription factors and intracellular molecules, including mitogen-activated protein kinases (MAPK) [20-22], nuclear factor-κB (NF-κB) [21] and nuclear factor-erythroid 2-related factor 2 (Nrf2) [22, 23]. Nrf2 is a redox-sensitive transcription factor that participates in the induction of phase II detoxifying and anti-oxidant proteins via the antioxidant-response element (ARE) [24, 25]. Nrf2 is sequestered in the cytoplasm by the inhibitory protein Keap1. Upon appropriate signals (i.e. oxidative stress), Nrf2 is released from Keap1 and translocates to the nucleus, thereby inducing the transcription of genes, including HO-1 [25].

We recently showed that 15d-PGJ₂ is a potent inducer of oxidative stress in human B cells [17]. The role of 15d-PGJ₂ in cytoprotective stress response, particularly in B cells, is unknown. Therefore, we hypothesized that 15d-PGJ₂ would increase the expression of HO-1 in human B-lineage cells via Nrf2. Herein, utilizing normal and malignant human B cells, we investigated (1) whether B cells can express HO-1; (2) the ability of 15d-PGJ₂ and known B cell activators to induce HO-1 expression; (3) whether or not HO-1 induction involves Nrf2; and (4) whether or not Nrf2 absence has an effect on normal and malignant B cells. In this report, we describe new findings on the molecular regulation of HO-1 and new insights into HO-1 expression in B cells.

Materials and Methods

Reagents

15d-PGJ₂ was purchased from Biomol (Plymouth Meeting, PA). Hemin and cycloheximide were obtained from Sigma (St Louis, MO). PGD₂, PGF_{2α} and CAY10410 were purchased from Cayman (Ann Arbor, MI). All prostaglandins, CAY10410 and Hemin were prepared in DMSO. Rabbit anti-human F(ab')₂ anti-IgM Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). CpG oligodeoxynucleotides 2395 (5'-TCGTCGTTTTTCGGCGCGCGCCG-3') is from Coley Pharmaceutical Group (Wellesley, MA). Recombinant human CD40L is from Alexis Biochem (Switzerland). Pokeweed mitogen (PWM) was a gift from Dr. S. Crotty and pansorbin (*Staphylococcus aureus* Cowen I strain) is from Calbiochem (La Jolla, CA). Actinomycin D was purchased from G Biosciences (Maryland Heights, MO) and LPS (*E. coli*) from Sigma (St. Louis, MO). siRNA against Nrf2 and siRNA control were purchased from Santa Cruz (Santa Cruz, CA).

Human Peripheral blood B lymphocyte and mouse B cell isolation

One unit (~500 ml) of blood was obtained from healthy donors as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection. All donors gave their consent prior to their inclusion in the study. B lymphocytes were isolated as previously described [26]. The Nrf2 knockout mouse strain (C57BL/6J background, No. 01390) used in this study has been described previously [27] and was generously supplied by Prof. Masayuki Yamamoto, University of Tsukuba, Japan via the RIKEN BioResource Center, Tsukuba, Japan. Control mice (C57BL/6) were purchased from the Jackson laboratory (Bar Harbor, ME). The mouse protocols were approved by The Animal Care and Use Committee of the University of Rochester. Mouse spleens were harvested, brought to single cell suspension and B cells were isolated using a CD19+ magnetic beads kit (Miltenyi, Auburn, CA) as recommended by manufacturer. The purity of isolated human and mouse B cells was assessed on a Becton-Dickinson FACSCalibur (BD Biosciences, Mountain View, CA) utilizing an antibody against CD19; B cell purity was > 95% (data not shown).

Mice and human cell culture

Human peripheral blood B cells, mouse B lymphocytes, Granta (mantle B cell lymphoma) and 8226 (multiple myeloma) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FBS, 50 μ M β -mercaptoethanol (Eastman Kodak, NY), 10 mM Hepes (U.S. Biochemical), 2 mM L-glutamine (Invitrogen Life Technologies), and 50 μ g/ml gentamicin (Invitrogen Life Technologies).

Western blot

Cells were harvested at the end of each treatment and resuspended in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 50 mM Tris (pH=8) and 0.1 % SDS). Nuclear proteins were obtained using a nuclear extraction kit (Active Motif, Carlsbad, CA). Ten μ g of total protein was loaded on a 12% SDS-PAGE gel, transferred to an Immun-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and blocked in 5% non-fat powdered milk in PBS with 0.1% Tween-20. Antibodies against HO-1 (1: 3000), HO-2 (1:5000; Stressgen Bioreagents, Victoria, BC), Nrf2 (R&D Systems, Minneapolis, MN and Sigma), Cox-2 (Cayman, Ann Arbor, MI) and actin (1:20000; Oncogene Research Products, San Diego, CA) were diluted in PBS containing 5% non-fat milk plus 0.1% Tween-20. Changes in protein levels were detected using Western Lightning Chemiluminescent PLUS Reagent (Perkin Elmer, Waltham, MA).

HO-1 immunofluorescence

8226 multiple myeloma cells were cultured for 24h in the presence of DMSO or 10 μ M 15d-PGJ₂. Cells were cytospun onto glass slides and fixed with 4% paraformaldehyde for 10 minutes at RT. Cells were then permeabilized in 0.3% Triton X-100 at RT for 10 minutes. Cells were blocked with 1% delipidized human serum for one hour at RT and then incubated with the anti-HO-1 antibody or non-immune isotype control (1:1000 in PBS plus 0.1% Tween-20) overnight at 4°C. Following this, the slides were incubated with a goat anti-rabbit FITC conjugated antibody (1:1000 in PBS plus 0.1% Tween-20) for one hour at RT, coverslipped in Immu-mount (Shanndon, Pittsburgh, PA) and viewed using an Olympus BX51 microscope (New Hyde Park, NY). Pictures were taken with a SPOT camera and analyzed using SPOT RT software (New Hyde Park, NY).

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from normal and malignant B cells using the RNeasy kit (Qiagen, Valencia, CA) and was reverse-transcribed using the First Strand cDNA synthesis kit

(Invitrogen) according to the manufacturer's protocol. This cDNA (1.5 μ l) was used as a template for HO-1 and HO-2 mRNA amplification using a PTC-200 thermal cycler (MJ Research Inc; Watertown, MA). Primer sequences were described previously [28]. 7S was used as housekeeping gene: 5'-ACCACCAGGTTGCCTAAGGA-3' (forward) and 5'-CACGGGAGTTTTGACCTGCT-3' (reverse). Annealing temperatures were: 55°C for 7S (25 cycles), 65°C for HO-1 (35 cycles) and 62°C for HO-2 (35 cycles). The sizes of the PCR products are 60 bp (7S), 270 bp (HO-1) and 1135 bp (HO-2). The products were resolved on agarose gel and visualized by ethidium bromide.

Nrf2 small interfering RNA (siRNA) cell transfection

8226 (multiple myeloma) cells were plated at 2×10^5 cells/well in a 6-well plate. Cells were transfected with Nrf2 or control siRNA as recommended by the manufacturer. Transfected cells were washed in culture media and exposed to 15d-PGJ₂ (10 μ M) for 24h, after which Nrf2 and HO-1 expression was assayed.

IgM and IgG enzyme-linked immunosorbent assays (ELISA)

Mouse plasma obtained from C57BL/6 and Nrf2^{-/-} mice by retro-orbital bleeding was used for determining IgM and IgG levels. Highly purified mouse B cells (0.5×10^6 cells/ml) were plated in 96-well round bottom-plates and were activated with LPS (1 μ g/ml) for 5 days. Supernatants were harvested and used for antibody ELISA assay. IgM and IgG was determined using the mouse-specific ELISA kit (Bethyl Laboratories) as recommended by the manufacturer.

Statistical analysis

One-way ANOVA, followed by Tukey post-hoc test were used for statistical analysis of the p value and values of $*p < 0.05$ are considered significant. Error bars represent the standard deviation of the mean (n=3).

Results

15d-PGJ₂ induces HO-1 protein expression in human B lymphocytes and malignant B cells

Whether or not HO-1 can be expressed by human B cells is unknown. Therefore, B cells were isolated from healthy donors and cultured for 24 h without (untreated) or with 15d-PGJ₂ (10 μ M). Both freshly-isolated and untreated B cells expressed little HO-1 (Figure 1A). However, treatment with 15d-PGJ₂ strongly induced HO-1. HO-2 was constitutively expressed (Figure 1A). Activation of B cells *in vitro* with a combination of classic stimuli such as anti-IgM, CpG, pokeweed mitogen (PWM), CD40L and pansorbin failed to increase HO-1 (Figure 1B).

HO-1 expression is high in some malignant non-hematopoietic cells [4]. However, there is no information regarding the expression of HO-1 in malignant human B cells. Therefore, we analyzed HO-1 expression in two different malignant B cells: Granta (mantle B cell lymphoma) and 8226 (multiple myeloma). Multiple myeloma 8226 cells exhibited basal HO-1 levels (Figure 2A). Exposure to 15d-PGJ₂ strongly induced HO-1 expression in both Granta and 8226 cells (Figure 2A). Hemin, a known inducer of HO-1, also increased HO-1 expression in malignant B cells (Figure 2A). HO-2 expression was constitutive, and similar between Granta and 8226 cells.

To determine the threshold at which 15d-PGJ₂ induced HO-1, Granta (mantle B cell lymphoma) cells and 8226 (multiple myeloma) were exposed to increasing concentrations of 15d-PGJ₂ for 24 h and HO-1 protein expression levels assessed. As shown in Figure 2B, HO-1 was induced in a dose-dependent manner. HO-1 was detectable following treatment

with 1 μ M of 15d-PGJ₂. Treatment with 5 and 10 μ M 15d-PGJ₂ further increased HO-1 levels. Because 10 μ M 15d-PGJ₂ resulted in a maximum increase in HO-1 levels, 10 μ M 15d-PGJ₂ was used for all subsequent experiments. The induction of HO-1 by 15d-PGJ₂ occurred between 2 and 4 h in 8226 cells (Figure 2C). Here, 8226 (multiple myeloma) cells exhibited a dramatic increase in HO-1 expression by 4h; HO-1 expression further increased by 6 h, after which protein levels declined (Figure 2C). Compared to 8226 cells, both Granta and normal B lymphocytes exhibited a delayed expression in HO-1 protein. Granta cells showed a small, but detectable expression of HO-1 at 4 h followed by an increase by 6 h (Figure 2D). Normal B cells showed a very low expression of HO-1 protein at 12 h followed by an increased response at 24 h (Figure 2E).

HO-1 has been localized to both the cytosol [29, 30], as well as the nucleus [31]. To determine the subcellular localization of HO-1 in B lineage cells, 8226 cells were treated with 15d-PGJ₂ for 2 4h and HO-1 expression examined by immunofluorescence. Cells that were untreated showed little fluorescence, indicative of low basal HO-1 expression (Figure 3A). Treatment with 15d-PGJ₂ induced HO-1 which was predominantly localized to the cytoplasm (Figure 3B, arrows).

15d-PGJ₂, but not a non-electrophilic analogue (CAY10410), increases HO-1 in normal and malignant B cells

Many of the biological actions of 15d-PGJ₂ relate to its electrophilic properties [17]. To determine if the induction of HO-1 by 15d-PGJ₂ was due to its highly reactive nature, we exposed normal B lymphocytes and Granta cells to 15d-PGJ₂, PGD₂ (the precursor of 15d-PGJ₂) and 9,10-dihydro-15-Deoxy- $\Delta^{12,14}$ -PGJ₂ (CAY10410), an analogue of 15d-PGJ₂ which lacks the reactive electrophilic carbon [17]. Some cells were treated also with PGF_{2 α} . HO-1 expression was low in normal B lymphocytes exposed to PGF_{2 α} or CAY10410 (Figure 4A). PGD₂ induced a modest (~10-fold) increase in HO-1. Treatment with 15d-PGJ₂ increased HO-1 expression by ~50-fold in normal B lymphocytes (Figure 4A).

Exposure of Granta cells to either PGF_{2 α} or CAY10410 did not increase HO-1 expression (Figure 4B). However, treatment with PGD₂ resulted in HO-1 levels that were nearly 500-fold above untreated cells. Further, there was nearly a 700-fold increase in HO-1 expression when these cells were treated with 15d-PGJ₂. 8226 cells showed a more dramatic increase in HO-1 induction (Figure 4C and 4D). PGD₂ caused a ~ 1000-fold increase in HO-1 levels and 15d-PGJ₂ further increased HO-1 expression by ~2000 fold. These new findings indicate that it is the electrophilic nature of 15d-PGJ₂ which contributes to its ability to up-regulate HO-1 in B lineage cells.

15d-PGJ₂ increases HO-1 mRNA expression in a time- and concentration-dependent manner in normal and malignant B cells

We next analyzed HO-1 mRNA in normal B cells, Granta (mantle lymphoma) and 8226 (multiple myeloma) by RT-PCR. Resting B cells had undetectable HO-1 mRNA levels (Figure 5A). However, HO-1 mRNA increased in 15d-PGJ₂-treated cells by 4 h, after which HO-1 mRNA expression decreased. By contrast Granta (mantle lymphoma) and 8226 cells (multiple myeloma) displayed basal HO-1 mRNA, which was further up-regulated by exposure to 15d-PGJ₂ (Figure 5B and 5C). The peak of HO-1 mRNA expression occurred between 4-6 h. HO-2 mRNA was not altered by 15d-PGJ₂.

15d-PGJ₂-induced HO-1 expression is controlled at the transcriptional level in human malignant B cells

Previously, it was shown that 15d-PGJ₂ induced HO-1 expression takes place at transcriptional in human lymphocytes [21]. To determine if HO-1 protein expression was

regulated at the transcriptional or translational level in Granta (mantle cell lymphoma) and 8226 cells (multiple myeloma), cells were treated with either actinomycin D (an inhibitor of mRNA synthesis) or cycloheximide (CHX; an inhibitor of protein synthesis) for one hour followed by exposure to 15d-PGJ₂. Inhibition of mRNA synthesis with actinomycin D abolished both HO-1 mRNA and protein expression in Granta cells treated with 15d-PGJ₂ (Figure 6). CHX disrupted HO-1 protein production in Granta cells (Figure 6B), but had no effect on HO-1 mRNA levels (Figure 6A). This suggests that HO-1 expression in human malignant cells is regulated at the transcriptional level. Similarly, CHX and actinomycin D abolished HO-1 protein synthesis in 8226 cells (Figure 6C).

HO-1 expression is dependent on Nrf2 in human and mouse B cells

Regulation of HO-1 expression in non-hematopoietic cells is believed to be governed by several transcription factors, particularly Nrf2 and p38 MAPK [22-25, 32]. To determine if, in B lineage cells, HO-1 upregulation is dependent on Nrf2, a small interfering RNA (siRNA) against Nrf2 was used. 8226 cells (multiple myeloma) were treated with Nrf2 siRNA or siRNA control, followed by 15d-PGJ₂ treatment. Figure 7A shows that in 15d-PGJ₂-treated cells, there is a dramatic decrease in HO-1 levels in Nrf2 siRNA-transfected cells (Figure 7A). These results demonstrate that 15d-PGJ₂-induced HO-1 expression is controlled by Nrf2 in human 8226 cells.

In order to determine whether or not Nrf2 is required for 15d-PGJ₂-dependent HO-1 induction in normal B cells, we used spleen B cells isolated from Nrf2-deficient mice or wild-type C57BL/6 controls. These cells were exposed *in vitro* to vehicle (DMSO) or 15d-PGJ₂ (10 μ M) for 24 h, after which HO-1 expression was analyzed. B cells isolated from control mice showed a strong induction in HO-1 expression in response to 15d-PGJ₂, whereas those from Nrf2-deficient mice did not express HO-1 protein in the presence of 15d-PGJ₂ (Figure 7B; compare lane 2 to lane 4). Administration of lipopolysaccharide (LPS) can cause an acute inflammation in mice. It was reported that Nrf2^{-/-} mice show a deregulated immune response during sepsis and an increase in pro-inflammatory genes expression [33]. One of the genes up-regulated by LPS is Cox-2. Cox-2 is considered a multifaceted protein [34]; Cox-2, via PGE₂ production, promotes inflammation but on the other hand via synthesis of 15d-PGJ₂ Cox-2 can also participate in the resolution of inflammation [16]. Furthermore, our laboratory reported that Cox-2 is actively involved in many B cell functions [35]. Therefore, we analyzed Cox-2 protein expression in B cells of Nrf2-deficient mice. Figure 7B shows that compared to B cells isolated from control C57BL/6 mice (lane 2), B cells from Nrf2^{-/-} mice exposed to 15d-PGJ₂ showed a heightened expression of Cox-2 (lane 4).

Next, we analyzed the antibody levels in plasma of naïve control and Nrf2^{-/-} mice. Figure 7C and 7D show that naïve Nrf2^{-/-} mice have an increased IgM and IgG level compared to naïve control mice. In order to see if there is a change in antibody production in activated B cells, highly purified mouse B cells were exposed to nothing or to LPS, a known B cell activator (1 μ g/ml), for 5 days and antibody production was determined by ELISA. Untreated B cells from normal and Nrf2^{-/-} mice showed no difference in IgM levels. However, IgG production was modestly increased in Nrf2^{-/-} B cells. LPS-activated B cells from Nrf2-deficient mice produced similar amounts of IgM (Figure 7E) and IgG (Figure 7F) compared to normal B cells.

Discussion

The prostaglandin 15d-PGJ₂, can be produced by mast cells, T lymphocytes and macrophages, and is proposed to be a key immunoregulatory mediator [16, 21]. 15d-PGJ₂ is formed spontaneously from PGD₂ and exerts some of its biological actions by inducing

ROS and the subsequent upregulation of proteins designed to combat oxidative stress [36]. HO-1 is a key cellular protein that confers cytoprotection [37, 38] and defends against inflammation [39]. The ability of a cell to withstand chronic oxidative stress and survive may contribute to the purported role of HO-1 in facilitating tumor progression [40, 41]. Although HO-1 expression has been described in mononuclear cells (a mixture of T cells, B cells and monocytes) [21], its expression in normal and malignant B lineage cells is unknown. We recently reported that B cells produce ROS upon exposure to 15d-PGJ₂ [17]. We therefore hypothesized that human B cells, exposed to 15d-PGJ₂, would increase HO-1 expression. Herein, we show that exposure of normal and malignant B cells to 15d-PGJ₂ potently induces HO-1 protein (Figures 1 and 2). Moreover, the ability of 15d-PGJ₂ to increase HO-1 was due to its electrophilic properties, as CAY10410, a structural 15d-PGJ₂ analog which lacks the electrophilic carbon, failed to increase HO-1 expression (Figure 4).

Many non-hematopoietic cancer cells have elevated HO-1 expression [2-4, 42]. We found that malignant B cells (mantle and MM) exhibited basal HO-1 mRNA expression and increased HO-1 mRNA levels more quickly in response to 15d-PGJ₂ compared to normal B cells (Figure 5). The presence of basal HO-1 mRNA may indicate that malignant B cells are “primed” to respond, and can dramatically increase stress-associated proteins, such as HO-1, when faced with adverse cellular conditions. In addition, the magnitude of HO-1 protein induction by both PGD₂ and 15d-PGJ₂ was 10-50 fold higher in malignant B cells compared to normal B cells (Figure 4). Subtle alterations in the *HO-1* gene promoter may account for the differential expression of HO-1 between normal and malignant B cells. The (GT)_n (purine-pyrimidine-alternating sequence) repeat is the most frequent of the simple repeats scattered throughout the human genome [43, 44]. *HO-1* gene promoter polymorphisms influence the level of HO-1 expression, with shorter (GT)_n repeats (< 25) [45] being associated with higher HO-1 transcriptional activity [46]. It may be that similar HO-1 polymorphisms exist between normal and malignant B cells, which could account for the differential expression of HO-1 protein.

The HO-1 promoter contains binding sites for several transcription factors, including NF-κB, AP-1 and Nrf2 [47]. Of these, Nrf2 appears prominent [23, 32, 48]. There is nothing known regarding Nrf2 expression/activation in normal or malignant B cells. We began by first examining Nrf2 expression in human tumor B cells (mantle cell lymphoma and multiple myeloma). Our results show that Nrf2 is expressed by human malignant B cells and that Nrf2 is, mainly, responsible for HO-1 expression in malignant human B cells (Figure 7A). In order to determine whether or not Nrf2 is required for 15d-PGJ₂-dependent HO-1 induction in normal B cells, we isolated B cells from Nrf2-deficient mice and we showed that in the absence of Nrf2, 15d-PGJ₂ failed to induce HO-1 protein expression (Figure 7B).

Studies utilizing Nrf2-deficient mice demonstrated the importance of Nrf2 in both antioxidant gene expression [49-51] as well as immune response. In this regard it has been reported that Nrf2-deficient mice display altered levels of B-cell specific genes [52]. We show that plasma levels of IgM and IgG are 3-4 times higher in Nrf2^{-/-}, further implicating HO-1 and/or Nrf2 in antibody production. However, when B cells were isolated from Nrf2^{-/-} mice they produced similar amounts of IgM and only modestly more IgG compared to control mice. Thus, *in vivo* global disruption of Nrf2 affects many cell types that could influence antibody production. Interestingly, we did find that Nrf2-deficient mice showed an increased expression of Cox-2 protein, even when unstimulated (Figure 7B). We previously reported that Cox-2 is critical for optimal antibody synthesis [35]. Therefore, it is possible that in the absence of Nrf2 increased Cox-2 levels in B cells may be responsible for the elevated antibody levels.

In summary, we report a higher level of expression of HO-1 by malignant B cells, compared to normal B cells, when provoked by 15d-PGJ₂, an anti-inflammatory prostaglandin. Moreover, the transcriptional regulation of HO-1 in B cells differs substantially from other cell types, in that Nrf2 alone is responsible for HO-1 expression. An intriguing finding was that when HO-1 expression was absent in Nrf2^{-/-} mice, Cox-2 expression was elevated. Therefore, we speculate that HO-1 expression may also help control Cox-2 levels. Overall, the new information presented herein regarding the ability of normal and malignant B lineage cells to strikingly upregulate HO-1, points to HO-1 as a possible key regulator of B cells. HO-1 may also confer a measure of cytoprotection to malignant B cells, as is postulated for non-hematopoietic cells [42, 53].

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A. Normal B cells **B. Normal B cells**

Figure 1. 15d-PGJ₂ induces HO-1 protein expression in normal human B lymphocytes
 Normal peripheral blood B cells were purified from three healthy donors and exposed *ex vivo* to 10 μ M 15d-PGJ₂. Untreated cells received only the vehicle (DMSO). Freshly isolated B cells were used as a comparison. HO-1 and HO-2 protein expression were determined by Western blot analysis. Total actin was used as a loading control. **A.** HO-1 expression is low in untreated B cells, but increased upon exposure to 15d-PGJ₂ in all three donors. HO-2 protein levels were constitutive. **B.** Activated B cells do not express HO-1. Normal B cells were exposed for 24 h to a combination of mitogenic agents: anti-IgM (2 μ g/ml) + CpG 2395 (1 μ g/ml); anti-IgM (2 μ g/ml) + CD40L (50 ng/ml); PWM (1:1000) + pansorbin (1:1000). HO-1 protein expression was upregulated only by 15d-PGJ₂. The Western blot shown is representative of 3 separate experiments.

Figure 2. 15d-PGJ₂ induces HO-1 expression in malignant human B cells

Granta (mantle lymphoma) and 8226 (multiple myeloma) cells were exposed to 10 μM hemin (positive control) or 10 μM 15d-PGJ₂ for 24h. Untreated cells received only the carrier (DMSO). HO-1 and HO-2 protein levels were determined by Western blot. **A.** Hemin and 15d-PGJ₂ induced HO-1 protein in Granta (mantle lymphoma) and 8226 (multiple myeloma). **B.** There was a concentration-dependent increase in HO-1 protein in 15d-PGJ₂ – treated Granta and 8226 cells. HO-1 protein levels increased in response to 1 μM 15d-PGJ₂. HO-1 further increased with treatment of 5 and 10 μM 15d-PGJ₂. 8226 cells, Granta and normal B lymphocytes were exposed to 15d-PGJ₂ (10 μM) and HO-1 expression was detected at different time points. There was a time-dependent increase in HO-1 protein expression in 15d-PGJ₂-treated 8226 cells (**C**), Granta (**D**) and normal B lymphocytes (**E**).

Figure 3. Cytoplasmic expression of HO-1 in multiple myeloma cells

8226 cells were untreated (DMSO) or treated with 10 μ M 15-PGJ₂ and HO-1 expression examined via immunofluorescence. **A.** Untreated cells exhibited negligible HO-1 expression. **B.** HO-1 protein exhibited predominantly cytoplasmic expression in 15d-PGJ₂-treated 8226 cells (arrows). Images shown are representative of 3 independent experiments.

Figure 4. 15d-PGJ₂, but not its non-electrophilic analogue (CAY10410), increased HO-1 in normal and malignant B cells

Normal and malignant B cells were exposed to 10 μ M of PGF_{2 α} , PGD₂, CAY14010 or 15d-PGJ₂ for 24h. Ten μ g of total protein was resolved on SDS-PAGE and HO-1 protein expression was analyzed by Western blot. Densitometric analysis was performed and graphs depict fold-induction in HO-1 protein compared to untreated controls (first column in Figure 4A, and 4C; untreated controls were run on the same gel); values were normalized to actin. **A.** 15d-PGJ₂ increased HO-1 expression approximately 50-fold in normal peripheral B cells. In contrast, the non-electrophilic derivative CAY10410 or PGF_{2 α} barely induced HO-1 compared to untreated cells. Normal B cells also showed an $\approx$ 10-fold increase in HO-1 protein when treated with PGD₂. **B.** Granta cells showed a dramatic increase in HO-1 protein after both PGD₂ treatment (500-fold) and 15d-PGJ₂ (700-fold increase) (compare to A). Induction of HO-1 by CAY10410 was minimal. **C and D.** In 8226 cells, PGD₂ and 15d-PGJ₂ increased HO-1 protein levels by $\sim$ 1000-fold and $\sim$ 2000 fold, respectively. Western blots are representative of 3 independent experiments.

A. Normal B cells **B. Granta (mantle lymphoma)** **C. 8226 (multiple myeloma)** **Figure 5. HO-1 mRNA profile in 15d-PGJ₂-treated human B cells**

Human peripheral B cells, Granta (mantle lymphoma) and 8226 (multiple myeloma) cells were exposed to 10 μ M 15d-PGJ₂ for 2-8 h and HO-1 and HO-2 mRNA levels were assayed by RT-PCR. 7S was used as a normalization control. There was a time-dependent increase in HO-1 mRNA upon exposure to 15d-PGJ₂ in normal B cells (A), as well as Granta (B) and 8226 (C). Note that both Granta (B) and 8226 (C) cells exhibited basal HO-1 mRNA expression.

Figure 6. 15d-PGJ₂-induced HO-1 synthesis is regulated at the transcriptional level
 Granta (mantle lymphoma) and 8226 cells (multiple myeloma) were pre-treated with actinomycin D or with cycloheximide (CHX) for 1 h followed by 15d-PGJ₂ treatment for 6 h (RT-PCR for Granta cells) or 18 h (Western blot for Granta and 8226 cells) and HO-1/HO-2 mRNA and protein expression were assayed. **A.** Actinomycin D inhibited 15d-PGJ₂-induced HO-1 mRNA expression in Granta cells. **B.** HO-1 protein expression was inhibited both by actinomycin D and CHX in 15d-PGJ₂-treated Granta and 8226 cells.

Figure 7. The Nrf2 transcription factor is required for 15d-PGJ₂-induced HO-1 expression in B cells but not for B cell antibody synthesis

A. 8226 multiple myeloma cells were exposed to Nrf2 or control siRNA (final concentration 10 nM) for 12 h in serum-free media after which cells were plated in fresh media and cultured for an additional 24h. Nrf2 siRNA and control cells were treated with 15d-PGJ₂ (10 μ M) for 24 h after which total cell lysates were isolated and Nrf2 and HO-1 protein levels were assayed by Western blot. Nrf2 protein levels increased after 15d-PGJ₂ treatment in the siRNA control cells (control siRNA). In Nrf2 siRNA- transfected cells, Nrf2 was knocked-down (compare lanes 3 and 4). Impressively, in Nrf2 siRNA- transfected 8226 cells, 15d-PGJ₂ induced only minimal HO-1 protein levels compared to siRNA control. **B.** Highly purified B cells from C57BL/6 or Nrf2^{-/-} mice were exposed to 15d-PGJ₂ (10 μ M) for 24 h. Western blot analysis of HO-1 protein shows that HO-1 expression was induced by 15d-PGJ₂ only in Nrf2-expressing C57BL/6 mice. In Nrf2^{-/-} mice, HO-1 expression was not detectable. Nrf2^{-/-} mice showed an increase in Cox-2 protein expression compared to control mice. **C** and **D.** Nrf2^{-/-} mice showed an increase in IgM and IgG in plasma compared to C57BL/6 controls. **E.** and **F.** Highly purified B cells from C57BL/6 or Nrf2^{-/-} mice were

stimulated with LPS (1 $\mu\text{g/ml}$) for 5 days after which IgM and IgG synthesis was determined by ELISA. Mouse experiments were done using 4 Nrf2^{-/-} and 4 C57BL/6 mice. One-way ANOVA followed by Tukey post-hoc test was used for statistical analysis and p values of * $p < 0.05$ is considered statistically significant.