Membrane Cholesterol Regulates Vascular Endothelial Cell Viability, Function, and Lipid Signaling

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Dedication

I wholeheartedly dedicate this work to my parents for their continued care, love, and encouragement throughout my life.

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Abstract

Membranes, including the plasma membrane, consist of phospholipids and cholesterol, which dictate their fluidity and the structural and functional integrities of the mammalian cells. Membrane cholesterol, especially the lipid raft-associated kind, has been emerging as one of the pivotal players among membrane lipids in modulating the cellular signaling cascades, which regulate normal cellular functions and pathophysiological events. Endothelial cells (ECs) of blood vessels are critical in the maintenance of the vascular tone, homeostasis of the blood vessel, and proper function of the cardiovascular system. Cholesterol of the vascular ECs is being recognized as an important element in the vascular EC signaling that dictates cellular replication and cell death. **Therefore, here, we hypothesized that the membrane cholesterol would be a key player in maintaining the viability, functions, and lipid signaling of the vascular ECs.** In our preliminary studies, to test our hypothesis, we had chosen three different widely used ECs in culture, including the bovine pulmonary artery ECs (BPAECs), bovine lung microvascular ECs, and human umbilical vein ECs as the model ECs. We then subjected these cells to the removal of membrane cholesterol by the treatment of two well established cholesterol-depleting agents, cyclodextrins, methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPCD) and had chosen that the BPAECs would be used as the model ECs for the entire study. Following the treatment of BPAECs with two different widely used concentrations of MβCD and HPCD (2% and 5%, wt/vol to deplete cellular cholesterol) for 15-120 min, the loss of membrane cholesterol, cell viability, and cell morphology were determined. Membrane cholesterol was determined in the cells by the spectrofluorometric method. Lactate dehydrogenase (LDH) released into the medium was determined by the spectrophotometric method. Cell morphology was examined by light microscopy. The results revealed that both

MβCD and HPCD caused significant and striking dose- and time-dependent loss of membrane cholesterol, loss of cell viability, and altered cell morphology in all the three chosen EC systems including the BPAECs. However, MβCD appeared to cause greater loss of membrane cholesterol, cell viability, and cell morphology as compared to the extent of the same caused by HPCD, under identical conditions, in all the chosen EC systems including the BPAECs. On the other hand, filipin (1-10 μM; as positive control), a fungal natural product, which sequesters but does not remove membrane cholesterol, caused only loss of cell viability in BPAECs without inducing the loss of membrane cholesterol. In conclusion, the results of this study showed that the membrane cholesterol was an important player in maintaining the cell viability and morphology in the vascular ECs. Removal of cholesterol by cyclodextrin (especially MβCD) treatment, apparently caused loss of fluidity of the cell membrane and leakage of vital cellular components, and thus caused loss of cell morphology in BPAECs. Also, the study offered a safer method of removal of cholesterol by utilizing HPCD, without causing extensive loss of cell viability as seen with the MβCD treatment, for studies to investigate the role of lipid raft-associated cholesterol in cellular functions. Phospholipases play important roles in cellular signaling during normal and pathophysiological states. Phospholipase D (PLD) is one such cell signaling phospholipase, which generates potent bioactive lipids such as phosphatidic acid, diacylglycerol, and lysophosphatidic acid from the membrane phospholipids. **Here, we also hypothesized that membrane cholesterol (including the raft-associated form) would also modulate the lipid signaling PLD in the vascular ECs.** Hence, we tested the modulatory effects of MβCD- and HPCD-assisted cholesterol removal on the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced (through protein kinase C [PKC] activation), diperoxovanadate (DPV)-induced (through protein tyrosine kinase [PTyK] activation), and oxidant (H_2O_2)-induced PLD

activation in BPAECs. Our results revealed that the two chosen cyclodextrins (MβCD and HPCD), differentially regulated the TPA-, DPV-, and oxidant-induced activation of PLD in BPAECs, suggesting that PLD responded to the differential removal of cholesterol and cellular effects (activation of upstream signaling kinases, PKC and PTyK) induced by MβCD and HPCD. Overall, this study established the importance of membrane cholesterol in vascular EC integrity and lipid signaling.

KEYWORDS: membrane cholesterol; lipid rafts; oxidant lipid signaling; phospholipase D; cyclodextrins; vascular endothelial cells

INTRODUCTION

Cell membranes including the plasma membrane host highly organized microdomains called lipid rafts which contain diverse signal transduction mediators (***1***). Lipid raft-associated cholesterol is a pivotal player among membrane lipids in modulating the cellular signaling cascades, which regulates normal cellular functions and pathophysiological events (***2***). Although the distribution of cholesterol in various cellular membranes has been found to be heterogeneous, it has been estimated that 80-90% of the entire cellular cholesterol is localized in the plasma membrane (***3***). Endothelial cells (ECs) of blood vessels are critical in the maintenance of the vascular tone, homeostasis of the blood vessel and proper function of the cardiovascular system. Cholesterol of the vascular ECs is being recognized as an important element in the vascular EC signaling that dictates cellular events including replication and cell death (***4, 5***). Among several key cellular signaling enzymes, phospholipase D (PLD), which generates potent bioactive lipid signal mediators such as phosphatidic acid (PA), diacylglycerol (DAG), and lysophosphatidic acid (LPA), has been recognized as one of the important players in cellular signaling events (***6***). PLD in vascular endothelial cells (ECs) has been shown to be activated by oxidants and oxidative stress through the modulation by protein kinase C (PKC), protein tyrosine kinases (PTyK), G proteins, thiol-redox regulation, and other complex cellular signaling mechanisms (***6***). Hence, from our preliminary studies with the bovine lung microvascular ECs, human umbilical vein ECs, and bovine pulmonary artery ECs (BPAECs), we selected BPAECs as the cell model. We then subjected the BPAECs to the removal of cholesterol with the aid of widely used cholesterol-depleting agents (cyclodextrins) and established a safer and effective method to deplete the lipid raft-associated cholesterol in order to study its role in the PKC- and PTyK-regulated and oxidant-induced PLD activation. However, it is increasingly evident that the lipid

signal enzymes are regulated by the lipid raft-associated cholesterol. Needless to mention, the methods of depletion of the raft-associated cholesterol have not been standardized in the vascular ECs in order to study the role of raft-associated cholesterol on the kinase-regulated and oxidant-induced PLD activation in ECs. The removal of the raft-associated cholesterol molecule from cells using the widely used cyclodextrins has been shown to be effective (***7***).

Our study focused on two of the β-cyclodextrin compounds, methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPCD) (**Fig. 1**). The BPAECs were treated for 60 min with 2% (wt/vol) concentration of the chosen cyclodextrins. Differences in the loss of cholesterol, cell viability, mitochondrial function, cell replication, cell morphology and 12-O-tetradecanoylphorbol-13-acetate (TPA)-, diperoxovanadate (DPV)-, and oxidant (H_2O_2)-induced PLD activation were determined. The results revealed that both MβCD and HPCD caused loss of cholesterol, loss of cell viability, and altered cell morphology in the chosen BPAEC system. However, MβCD appeared to cause greater loss of cholesterol, cell viability, and cell morphology as compared to the extent of the same caused by HPCD, under identical conditions, in the chosen EC system. Also, the study demonstrated that removal of cholesterol by MβCD caused enhanced activation of PLD in BPAECs, which was further potentiated by the oxidant (100 µM H_2O_2) treatment. However, depletion of cholesterol by HPCD alone did not cause the activation of PLD in BPAECs, but resulted in the oxidant (100 µM H_2O_2)-induced PLD activation. In comparison with HPCD, cholesterol depletion by MβCD resulted in a greater extent of the oxidant-induced PLD activation in BPAECs. Nevertheless, our study also demonstrated that the MβCD treatment caused greater adverse cellular effects than those caused by HPCD treatment, although both MβCD and HPCD were effective in removing the raft-associated cholesterol including the plasma membrane cholesterol in BPAECs.

In conclusion, the results of this study showed that the cholesterol was an important player in maintaining the cell viability and morphology in the vascular ECs. Removal of cholesterol by cyclodextrin (especially MβCD) treatment, apparently caused loss of fluidity of the cell membrane and leakage of vital cellular components, and thus caused loss of cell morphology in BPAECs. Also, the study offered a safer method of removal of cholesterol by utilizing HPCD, without causing extensive loss of cell viability as seen with the MβCD treatment, for studies to investigate the role of lipid raft-associated cholesterol in cellular functions such as lipid signaling. Overall, this study emphasizes the importance of membrane cholesterol in vascular EC integrity and functions.

MATERIALS AND METHODS

Materials

Bovine pulmonary artery endothelial cells (BPAECs) (passage 4) were commercially obtained from VEC Technologies (NY, USA). Minimal essential medium (MEM), nonessential amino acids, trypsin, fetal bovine serum (FBS), penicillin/streptomycin, DMEM phosphate-free modified medium, phosphate-buffered saline (PBS) and Amplex Red Cholesterol Assay Kit were acquired from Invitrogen Corporation (Grand Island, NY). Methyl-β-cyclodextrin (MßCD), 2-hydroxypropyl-β-cyclodextrin (HPCD), and LDH assay kit was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Phosphatidylbutanol (PBt) was obtained from Avanti Polar Lipids (Alabaster, AL). [^{32}P] Orthophosphoric acid and [^{3}H] thymidine were procured from Perkin Elmer (Shelton, CT). Endothelial cell growth factor was purchased from Upstate (Millipore) (Charlottesville, VA). 12-O-tetradecanoylphorbol-13-acetate (TPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and H_2O_2 (3% or 0.88 M) were obtained from Fisher Scientific (Pittsburgh, PA). Uniplate, Silica Gel-H coated, thin layer chromatography (TLC) plates were purchased from Analtech (Newark, DE). Diperoxovanadate (DPV) has been kindly provided by Prof. T. Rama Sarma of the Indian Institute of Sciences, Bangalore, India.

Cell culture

BPAECs were grown to confluence (~95%) in MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, 5 µg/mL endothelial cell growth factor, and 1% nonessential amino acids at 37° C in a humidified environment of 95% air-5% CO_2 as described earlier in (***6,8***). BPAECs, from passages 4-15, were used in the experiments. BPAECs from each primary T-75 cm flask were detached with 0.05% trypsin, re-suspended in fresh

medium, and sub-cultured in 35-mm sterile dishes in complete medium to the required level of confluence under a humidified environment containing 95% air-5% CO_2 at 37º C for treatments with the desired agents.

Membrane Cholesterol Depletion and Determination

BPAECs in 35-mm dishes were grown up to 90% confluence. Cells were treated with basal MEM alone or basal MEM containing the chosen β-cyclodextrin (2% MβCD or HPCD, wt/vol) for 1 h at 37º C in a humidified environment of 5% CO_2 - 95% air. At the end of the incubation, the medium was removed and 1 mL of PBS was added to each dish containing the cells. The cells were then detached with a cell scrapper and transferred into an Eppendorf tube and centrifuged for 10 min at 15,000 RPM. After centrifugation, the supernatant was removed without disturbing the cell pellet. The cholesterol amount in the cells was then determined spectrofluorometrically according to the manufacturer's recommendations (Molecular Probes - Invitrogen Detection Technologies, Grand Island, NY).

Lactic Dehydrogenase (LDH) Assay of Cytotoxicity

BPAECs in 35-mm dishes were grown up to 90% confluence. Cells were treated with basal MEM alone or basal MEM containing the chosen β-cyclodextrin (2% MβCD or HPCD, wt/vol) for 1 h at 37º C in a humidified environment of 5% CO_2 - 95% air. At the end of the incubation, the medium was collected. The experiment was terminated with 1N HCl. LDH release was measured by the spectrophotometric method according to the manufacturer's recommendations (Sigma Chemical, St. Louis, MO) (***9***).

Cellular Morphology

Morphological changes in BPAECs grown in 35-mm dishes up to 90-100% confluence, following their exposure to different concentrations of MβCD and HPCD for 1 h, were examined as an index of cytotoxicity. Images of cellular morphology were digitally captured using the Nikon Eclipse TE2000-S at either 10 X or 100 X magnification.

MTT Assay of Cytotoxicity

BPAECs were grown up to 90% confluence in 17.5-mm dishes pretreated with MEM alone or MEM containing different concentrations of either MβCD or HPCD for 2 h. At the end of incubation period, MTT solution (10% of culture volume) was added and incubated for 3-4 h, the culture fluid was removed and MTT solvent was added in an amount equal to original culture volume. Absorbance was measured according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO).

[^{3}H] Thymidine Incorporation Assay for Cell Proliferation

BPAECs were grown to 70% confluence in 35-mm dishes. MβCD or HPCD treatments were added to the wells. The treatment media was then removed and 1 ml of [^{3}H]-thymidine (1 μCi/ml) in MEM was added to each well and incubated for 24 h. After incubations, [^{3}H]-thymidine was removed and cells were washed with PBS. Cells were then washed with 5% trichloroacetic acid (TCA) in distilled water, and then 500 μL of 10.25 M NaOH was added to each dish for 30 min. 400 μL of the cell solubilization solution was added to the scintillation

vials, and samples were analyzed using [^{3}H] radioactivity in [^{3}H] channel of the Packard Tri-carb 2900TR Liquid Scintillation Counter.

Phospholipase D (PLD) activation in intact ECs

BPAECs in 35-mm dishes ($5x10^{5}$ cells/dish) were pre-labeled with [^{32}P] orthophosphoric acid (5 µCi/mL) in DMEM phosphate-free medium containing 2% fetal bovine serum for 12-14 h (***6, 8***). Cells were washed with basal MEM and treated under the determined conditions with the chosen cyclodextrin compound. Cells were then incubated at 37° C in 1 mL of basal MEM containing 0.05% 1-butanol in absence and presence of TPA (25 nM) or DPV (10 µM) or H_2O_2 (100 µM) for the desired length of time under a humidified environment of 95% air-5% CO_2. The treatments were terminated by the addition of methanol:conc. HCl (100:1, by vol.). Lipids were extracted essentially according to the method of Bligh and Dyer procedure as described previously (***6, 8***). [^{32}P]-Labeled phosphatidylbutanol (PBt) formed from the PLD activation and transphosphatidylation reaction, as an index of PLD activity in intact cells, was separated by the TLC (***6, 8***). Radioactivity associated with the [^{32}P]-PBt was determined by the liquid scintillation counting, and data were expressed as DPM normalized to 10^{6} counts in the total cellular lipid extract.

Statistical analysis of data

Standard deviation (SD) for each data point was calculated from triplicate determinations under identical conditions. Data were subjected to one-way analysis of variance and pair-wise multiple comparisons were done by Dunnett's method with the significance set at $P<0.05$.

RESULTS

Treatment with the cyclodextrins, MβCD and HPCD, in vascular endothelial cells results in cholesterol depletion and increased cytotoxicity (Figs. 2-4)

Cholesterol is an important constituent of the mammalian cell membrane, mainly the plasma membrane. In order to study the role of lipid raft-associated cholesterol in cellular signaling events, the β-cyclodextrins, which offer the utility for solubilizing, have been widely utilized to remove or deplete cholesterol from mammalian cells (***11,12***). In the current study, both MβCD and HPCD were shown to be effective in depleting cholesterol from BPAECs, however, HPCD removed significantly less cholesterol than MβCD. MβCD **[Fig. 2A]** showed significant cholesterol loss at 15 min (5% concentration) and 60 min (2% contentration), where as HPCD **[Fig. 2B]** showed significant cholesterol loss at 15 min (both 2% and 5% concentrations). The respective amount of cholesterol removed using the MβCD treatment, however, was significantly greater than that of HPCD. Time- and dose-responses before these listed points were considered not significant either at or above determined control values. Although both compounds removed statistically significant levels of cholesterol, there were differences in the resulting LDH leak into the cell culture medium. Increased LDH leak is directly proportional to increased cytotoxicity. MβCD **[Fig. 3A]** showed significant LDH leak at 15 min (both 2% and 5% concentrations), whereas HPCD **[Fig. 3B]** only showed significant LDH leak at 120 min (5% concentration). Time- and dose-response before these listed points were considered not significant either at or below determined control values. These results were also confirmed upon examination of cellular morphology with light microscopy. Increased loss of cell morphology would cause an increase in leakage of vital cellular components. MβCD **[Fig. 4A]**, showed increased loss of cellular morphology as both incubation time and dose

progressed. HPCD **[Fig. 4B]** showed markedly less loss of morphology at the same dose and time points. It can be concluded from this data that, as compared to HPCD, MβCD caused a greater extent of cholesterol depletion, increased cytotoxicity, and severe loss of cellular morphology.

Cholesterol removal by MβCD and replenishment by cholesterol supplementation provides protection against cytotoxicity in vascular endothelial cells (Figs. 5-7)

After depletion with MβCD, supplementational effects with water-soluble cholesterol were examined. Corresponding with what was previously shown, after 60 min, cholesterol depletion with MβCD (2% and 5%) **[Fig. 5]** showed significantly decreased levels of cholesterol as compared to the control cells. Nevertheless, replenishment with water-soluble cholesterol increased measured cholesterol levels above control levels. The index of cytotoxicity of cholesterol replenishment was assessed by measuring levels of LDH leak into the liquid cellular environment **[Fig. 6]**. Increased LDH leak is directly proportional to increased cellular cytotoxicity. Cholesterol removal with MβCD (2%) for 60 min followed by replacement with basal MEM showed a significant increase in cytotoxicity at both 30 and 60 min after basal MEM replacement. Furthermore, MβCD (2%) depletion for 60 min followed by supplementation with water-soluble cholesterol (2%), demonstrated a significant reduction in cyclodextrin-induced cytotoxicity after 60 min of cholesterol rescue. These results were also confirmed upon examination of cellular morphology with light microscopy **[Fig. 7]**. Increased loss of cell morphology would cause an increase in leakage of vital cellular components. With a control set of water-soluble cholesterol (2%), no loss of morphology was expected or seen. After 60 min of cholesterol depletion with MβCD (2%), a significant loss of cell morphology was shown, as

expected from previously established studies. After 60 min of cholesterol replenishment, however, the cyclodextrin-induced loss of morphology was reversed, and morphology was restored to control established levels. In conclusion, after cholesterol depletion, increased cytotoxicity, and loss of morphology caused by MβCD (2%) were seen, viability was able to be rescued and returned to normal control levels by supplementing the vascular endothelial cells with water-soluble cholesterol (2%) for 60 min.

Cyclodextrin-induced inhibition of MTT reduction in vascular endothelial cells (Fig. 8)

To further assess the viability of vascular endothelial cells after dose-dependent cholesterol depletion with MβCD or HPCD (2% or 5%), cellular MTT reduction as an index of mitochondrial function was measured **[Fig. 8]**. Results show that after 60 min of cholesterol depletion with both cyclodextrins at both concentrations caused a statistically significant decrease in mitochondrial MTT reduction ability. Upon further analysis, however, it was seen that MβCD (both 2% and 5%) showed a markedly greater decrease in mitochondrial function as compared to the control cells treated with basal MEM alone. In addition, MβCD (both 2% and 5%) also showed a markedly greater decrease in MTT reduction as compared to HPCD (both 2% and 5%). In conclusion, this data confirmed that MβCD caused a greater loss of cellular viability, specifically in MTT reduction ability, as compared to the alternate cyclodextrin, HPCD.

MβCD-induced inhibition of [^{3}H]-thymidine incorporation and comparison with HPCD in vascular endothelial cells (Fig. 9)

Cellular replication was measured by determining the ability of BPAECs to incorporate [^{3}H]-thymidine into cellular DNA **[Fig. 9]**. Cholesterol was depleted from the cells in a dose-dependent manner with both MβCD and HPCD (2% and 5%). After 24 h of incubation with [^{3}H]-thymidine, MβCD (both 2% and 5%) showed a markedly significant decrease in replication and nucleotide incorporation, as compared to the control cells treated with basal MEM alone. HPCD (both 2% and 5%), however, did not show statistically significant decreases in replication or nucleotide incorporation. In addition, both concentrations of MβCD treated cells showed a significantly lower ability to incorporate [^{3}H]-thymidine as compared to HPCD-treated vascular endothelial cells. In conclusion, this data further confirmed that MβCD caused a greater loss of cellular viability, specifically replication ability, as compared to the alternate cyclodextrin, HPCD.

Dose-response and time-course treatment of MβCD- and HPCD-mediated cholesterol removal modulates TPA-induced (PKC-activated) PLD activity in vascular endothelial cells. (Figs. 10-11)

Phospholipase D (PLD), is an important lipid signaling enzyme, which exists in two isoforms, PLD_1 and PLD_2, in mammalian cells including the vascular endothelial cells **(6)**. Upon cleavage of a phospholipid in the cellular membrane, especially phosphatidylcholine, PLD forms phosphatidic acid, the bioactive lipid signal indicator **(6)**. However, cholesterol (plasma membrane and raft-associated) is know to modulate the structure and function of the biological membranes, and hence the cellular signaling and functions. PLD is not an exception to this. These results demonstrate the effect of cholesterol depletion on PLD activation signaling within vascular endothelial cells. TPA, which acts through the PKC pathway, was used to induce the

activation of PLD. Dose-dependent cholesterol removal by MβCD (0.5% and 1.0%) for 60 min, followed by a challenge with TPA (25 nM) for 60 min **[Fig. 10A],** showed an increase in PLD activation as compared to the control cells treated with basal MEM alone. In the same experiment, after 60 min, MβCD (2.0%), however, showed a slightly less extent of PLD activation after a 60 min TPA challenge as compared to the control cells. In comparison, dose-dependent cholesterol removal by HPCD (0.5%, 1.0%, and 2.0%) for 60 min, followed by a challenge of TPA (25 nM) for 60 min **[Fig. 10B]**, showed a statistically significant increase in PLD activation by TPA as compared to control cells treated with basal MEM alone. Using MβCD, PLD activation reached a peak level at the 0.5% concentration, whereas with HPCD, the peak activation was not seen until the 2.0% concentration. Both cyclodextrins were also analyzed in a time-dependent manner. Cholesterol removal with MβCD (0.5%) (30, 60, 120 min) followed by a challenge by TPA (25 nM) for 60 min, **[Fig 11A]** showed a statistically significant increase as the time progressed as compared to the control cells treated with basal MEM alone. Similar results were seen with cholesterol depletion by HPCD (0.5%) (30, 60, 120 min) followed by a challenge by TPA (25 nM) for 60 min, **[Fig 11B].** As time progressed in this experiment, PLD activation was statistically increased as compared to the control cells treated with basal MEM alone. In conclusion, PLD activation through the PKC-activated pathway (TPA-induced) was enhanced by cholesterol removal assisted by both MβCD and HPCD. MβCD, however, showed a greater and also quicker effect as compared to HPCD. In direct comparison of both β-cyclodextrins, PLD activation increased to an even greater extent when more cellular cholesterol was removed with MβCD.

Dose-response and time-course treatment of MβCD- and HPCD-mediated cholesterol removal modulates DPV-induced (PTyK-activated) PLD activity in vascular endothelial cells (Figs. 12-13)

A second mode of activation of PLD is through the PTyK pathway. An established activator of the PTyK pathway is DPV. Dose-dependent cholesterol removal by MβCD (0.5%) for 60 min, followed by a challenge with DPV (10 μM) for 30 min **[Fig. 12A]**, showed a decrease in PLD activation by DPV as compared to the control cells treated with basal MEM alone. In the same experiment, after 60 min, MβCD (1.0% and 2.0%), however, showed an increase in PLD activation after a 30 min DPV challenge as compared to the control cells. In comparison, dose-dependent cholesterol removal by HPCD (0.5%, 1.0%, and 2.0%) for 60 min, followed by a challenge of DPV (10 μM) for 30 min **[Fig. 12B]**, showed a statistically significant decrease in PLD activation by DPV as compared to control cells treated with basal MEM alone. Using MβCD, PLD activation reached peak levels at all tested concentrations, as compared to that caused by HPCD. Both cyclodextrins were also examined for a time-dependent response. Each time point of cholesterol removal with MβCD (0.5%) (30, 60, 120 min) followed by a challenge with DPV (10 μM) for 30 min, **[Fig 13A]** showed a statistically significant lower extent of PLD activation as compared to the control cells treated with basal MEM alone. As the time progressed, however, the extent of PLD activation increased. Similar results were seen with cholesterol depletion by HPCD (0.5%) (30, 60, 120 min) followed by a challenge with DPV (10 μM) for 30 min, **[Fig 13B]**. Once again, PLD activation was statistically lower in cyclodextrin treatments as compared to the control cells treated only with MEM followed by the DPV challenge. In conclusion, PLD activation through the PTyK-activated pathway (DPV-induced) was lowered by cholesterol removal assisted by both MβCD and HPCD. MβCD, however,

showed a greater and also quicker effect as compared to HPCD, especially in the time-course experiments. Hence, in direct comparison of both β-cyclodextrins, the activation increased to an even greater extent when more cellular cholesterol was removed.

Modulation of oxidant (H_2O_2)-induced activation of PLD by MβCD and HPCD-mediated cholesterol depletion in vascular endothelial cells (Fig. 14)

It has been previously established that PLD is activated by oxidants including H_2O_2 **(6, 8)**. Here we examined the effects of cholesterol depletion assisted by MβCD and HPCD on oxidant-induced PLD activation. The oxidant used in this study was hydrogen peroxide (H_2O_2), which can act through either the PKC or PTyK pathways to activate PLD in vascular endothelial cells. Cholesterol removal by both MβCD (2%) and HPCD (2%) for 60 min followed by a challenge with H_2O_2 (100 μM) for 60 min **[Fig. 14]** showed significant enhancement of PLD activation. In addition, MβCD (2%), as compared to HPCD (2%), showed a significantly greater extent of H_2O_2-induced PLD activation. These results indicated that the H_2O_2–induced PLD activation was enhanced by the cellular cholesterol removal assisted by the MβCD and HPCD compounds, wherein the effect of former cyclodextrin was greater than the effect of the latter. In direct comparison of both β-cyclodextrins, the enzyme activation was further enhanced when more cellular cholesterol was removed with MβCD.

DISCUSSION

This study revealed that cholesterol, especially the lipid-raft associated type, is essential for cellular viability and function. Furthermore, membrane cholesterol is a vital component in cellular signaling modulation, specifically the PLD activation pathway. In order to study the role of cholesterol in cellular viability, function, and signaling, two β-cyclodextrins were utilized as tools to remove cellular cholesterol from the BPAECs in this study. These compounds offer a utility for solubilizing dietary preparations, and have been widely utilized to remove or deplete cholesterol from mammalian cells (***11,12***). Among the β-cyclodextrins, MβCD appears to be a popular cyclodextrin that is being widely utilized in removing cholesterol from the plasma membrane and lipid rafts of the mammalian cells. Up to 2% (wt/vol) or 10 mM concentration of MβCD has been used in several reported studies to remove cholesterol from different mammalian cell models (***13-15***). Moreover, in several of these documented studies cells have been treated with MβCD for prolonged time periods up to 4 h. MβCD has also been identified to be a toxic compound (***11***). MβCD has been shown to disrupt lipid rafts and cause apoptotic cell death in keratinocytes (***13***).

In the current study, although MβCD was shown to be effective in depleting cholesterol from BPAECs, its cytotoxicity was inevitable. This effect was also shown through the alterations of cellular morphology as observed under light microscope. The less widely used cyclodextrin, HPCD, was shown to cause no cytotoxicity to BPAECs while effectively removing the cellular cholesterol. The alterations in cellular morphology due to HPCD treatment were markedly less drastic as compared to MβCD. Whether the effects of MβCD are solely due to its potential to remove cholesterol from cells or due to other cellular biochemical alterations/perturbations are not thoroughly clear. Nevertheless, it is emerging that MβCD

causes other cellular biochemical alterations such as the removal of membrane phospholipids, fatty acids, and proteins (Hinzey, unpublished observations), in addition to depleting EC cholesterol. In comparison to MβCD, HPCD appeared to be safer in not causing adverse cellular effects and efficiently removing cholesterol from the ECs. It is surmised that differences in the polarity of the two cyclodextrins (MβCD being less polar) could possibly aid in the different cellular effects of each compound.

In order to examine the extent of damage and its effect on the cellular viability, cholesterol was supplemented to the cholesterol-depleted cells following MβCD treatment. This replenishment was shown to replace depleted cholesterol, decrease cytotoxicity, and restore cellular morphology. From this data, it can be concluded that cholesterol replenishment with water-soluble cholesterol may restore cellular viability and function and cellular cholesterol (both the plasma membrane and raft-associated species) is crucial for the structure and function of the ECs.

Cell viability was further examined through the mitochondrial reduction of MTT. Both MβCD and HPCD showed a decrease in MTT reduction by BPAECs, but MβCD was markedly more drastic. In addition, cholesterol depletion with MβCD showed drastically less ability of BPAECs to incorporate [^{3}H]-thymidine as compared to HPCD. Inability of BPAECs to incorporate the [^{3}H]-thymidine into the cells may be attributed to their inability to successfully replicate cellular DNA. Therefore, removal of cholesterol, and leakage of other vital cellular components is more drastic when MβCD is utilized thus leading to a drastic decline of EC replication. The current results also suggested that MβCD-depleted cholesterol or MβCD treatment might have contributed to the loss of proper energy metabolism, maintenance of the

nucleotide pools, and damage to the nuclear machinery, all of which could have led to the loss of cellular replication in BPAECs.

PLD is an important cell-signaling enzyme, which is activated by various pathways controlled by complex signaling mechanisms (***6***). The enzyme, which exists in two isoforms, PLD_1 and PLD_2, in mammalian cells including the vascular endothelial cells, forms phosphatidic acid (PA), the bioactive lipid signal indicator, upon its action on the membrane phospholipids, especially, phosphatidylcholine (PC) **[Schema-1]**. However, cholesterol (plasma membrane and raft-associated) is known to modulate the structure and function of the biological membranes, and hence the cellular signaling and functions. PLD, a signaling phospholipase is not an exception to this. Studies established the activation of PLD through two different mechanisms such as activation by PKC and PTyK (***6***). In the current study, we followed three experimental approaches in order to study the effects of cyclodextrin-depleted cholesterol on the PKC-, PTyK-, and oxidant-activated PLD in BPAECs. Accordingly, PKC activation in cells was induced by TPA, and it was shown that cholesterol depletion, through both dose- and time-dependent mechanisms, increasingly led to PLD activation in BPAECs. The greatest extent of PLD activation varied for each cyclodextrin compound, appearing at a lower dose and earlier time point for MβCD. This can most likely be attributed to the observed profound detrimental effects of MβCD on BPAECs in the current study. Effectiveness of cholesterol removal can be viewed as a stoichiometric relationship. This may be explained as a small (certain stoichiometric) amount of removal of cholesterol still enables the activation of signaling pathways, whereas a larger amounts of cholesterol removal, and other vital components, causes inhibition of signaling pathways, which could be due to the decrease in cellular viability seen in cyclodextrin-treated cells as observed in the current study.

Peroxovanadium compounds such as DPV have been shown to cause the activation of PTyK which in turn activate PLD through protein tyrosine phosphorylation. Hence, in the current study, we used DPV as a tool to activate PTyK and then studied the effects of cyclodextrin-mediated cholesterol removal on the PTyK-activated PLD in BPAECs. These results were different from those seen with the TPA-induced and PKC-regulated PLD activation. Drastic cholesterol depletion, through both dose- and time-dependent, did show enhanced PLD activation, however these extents of enzyme activation were consistently less than the activation with DPV alone. These results also suggested that cholesterol depletion by cyclodextrins could have an inhibitory effect on the PTyK which then were not effective in activating PLD even after treatment with DPV. Thus, it appeared that cyclodextrin-assisted cholesterol removal had differential effects on PLD activation in ECs through the upstream signaling protein kinases. These effects could be from activating certain protein kinases (e.g. PKC) and/or inhibiting certain other protein kinases (e.g. PTyK), in the cellular phospholipid-cholesterol microdomains.

Our earlier studies have established that oxidants including H_2O_2 activate PLD in the vascular ECs through multiple upstream signaling pathways (***6***). Therefore, in the current study we investigated the effects of cyclodextrin-depleted cholesterol on the H_2O_2-induced activation of PLD in BPAECs. Oxidative PLD activation can occur through the activation of both the PKC and PTyK pathways and also by redox regulation (***6***). Our current study demonstrated that cholesterol removal by both MβCD and HPCD caused significant enhancement of the H_2O_2-induced PLD activation in ECs, with MβCD exerting a greater effect. However, MβCD alone, in comparison with HPCD caused significant activation of PLD as compared to that in the control cells. The enhancement of oxidant-induced PLD activation in BPAECs by the cyclodextrin-

assisted removal of cellular cholesterol could be due to the (1) activation of certain upstream protein kinases and/or (2) alterations in the cellular redox regulation.

Cholesterol is an important constituent of the mammalian cell membranes, mainly the plasma membrane. Being heterogeneously distributed among different membrane locations in the cell, the plasma membrane hosts up to 80-90% of the total cellular cholesterol (***3***), wherein it plays a major role in the organization and function of cellular lipid rafts which take part in signal transduction events (***10***). From this study, it is concluded that cholesterol is an important component of the vascular EC membrane and raft systems. Cyclodextrins can be used for the removal of cellular cholesterol (plasma membrane and raft-associated) in studies aimed at the role of lipid raft-associated cholesterol on the agonist- and oxidant-activated lipid signaling enzymes in ECs, provided the chosen cyclodextrin is not cytotoxic and only effective in cellular cholesterol removal **[Schema-2]**.

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FIGURE LEGENDS

Fig. 1. *Structures of cyclodextrins, MβCD and HPCD used for cholesterol depletion*

Structural representations of methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPCD).

Fig. 2. *Cholesterol depletion by MβCD and HPCD in vascular endothelial cells in a time- and dose-dependent manner*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated for 15, 30, 60, or 120 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (2% or 5% wt/vol). The amount of cholesterol (µg) in cells following the treatments was determined spectrofluorometrically as described in the Methods section. This figure illustrates how both β-cyclodextrin compounds are effective in removing the cholesterol from the BPAECs; however MβCD removed far more cholesterol in comparison with HPCD at both concentrations. Data represent mean±SD of three independent experiments. *Significantly different at $P<0.05$ as compared to the control cells treated with basal MEM alone.

Fig. 3. *MβCD- and HPCD-induced cytotoxicity in vascular endothelial cells in a time- and dose-dependent manner*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated for 15, 30, 60, or 120 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (2% or 5% wt/vol). LDH released is directly proportional to the extent of cytotoxicity and indicative of the cell viability. The extent of LDH release was analyzed spectrophotometrically using the LDH Assay Kit as described in the Materials section. This figure demonstrates the cytotoxicity caused by MβCD starting at the

30 min treatment, while HPCD at the 120 min treatment. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone.

<u>Fig. 4.</u> *MβCD and HPCD-induced cellular morphology alterations in vascular endothelial cells in a time- and dose-dependent manner*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or MEM containing MβCD **[A]** or HPCD **[B]** (2% or 5% wt/vol) for 15, 30, 60, or 120 min as described in the methods section. At the end of the treatment time, cells were examined under light microscopy at 10X or 100X magnification. Images were captured digitally. Each micrograph is representative of three independent observations.

<u>Fig. 5.</u> *Cholesterol removal by MβCD and replenishment by cholesterol supplementation in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or MEM containing MβCD (2% or 5%) for 60 min. The cells were then challenged with water-soluble cholesterol (2% or 5%) for 60 min to replenish cyclodextrin-depleted cellular cholesterol. At the end of the treatment time, cellular cholesterol was determined as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone. **Significantly different from MβCD-treated cells at P<0.05.

Fig. 6. *Protection against cytotoxicity induced by cyclodextrin-mediated cholesterol removal by cholesterol replenishment in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or MEM containing MβCD (2%) for 60 min, following which they were supplemented with water-soluble cholesterol (2%) for 30 and 60 min. At the end of both challenge times, the LDH release into the medium was assayed as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone. **Significantly different from MβCD-treated cells at P<0.05.

Fig. 7. *Rescue of cyclodextrin-induced cell morphology alterations by cholesterol replenishment in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or MEM containing MβCD (2%) for 60 min, following which they were supplemented with water-soluble cholesterol (2%) for 60 min. At the end of the treatment time, cellular morphology was examined by light microscopy as described in the Methods section at 100X magnification. Images were captured digitally. Each micrograph is representative of three independent observations.

Fig. 8. *Cyclodextrin-induced inhibition of MTT reduction in vascular endothelial cells*

Confluent BPAECs ($2x10^5$ cells/17.5-mm dish) were treated with basal MEM or MEM containing MβCD or HPCD (2% or 5% wt/vol) for 60 min, following which cellular MTT reduction was assayed as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone.

Fig. 9. *MβCD-induced inhibition of [^{3}H]-thymidine incorporation and comparison with HPCD in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or MEM containing MβCD or HPCD (2% or 5%) for 60 min, following which the medium was removed and cells were incubated with [^{3}H]-thymidine (1 μCi/ml) for 24 h. The cell replication at the end of the treatment was assayed by determining [^{3}H]-thymidine incorporation into the cells as outlined in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at $P<0.05$ as compared to the control cells treated with basal MEM alone.

Fig. 10. *Dose-response of MβCD- and HPCD-mediated cholesterol removal modulation of TPA-induced (PKC-activated) PLD activity in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated for 60 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (0.5%, 1%, or 2% wt/vol). Following cholesterol removal from cells, the treatments were removed and the cells were treated with basal MEM only or basal MEM containing TPA (25 nM) for 60 min and the PLD activity was determined as the formation of [^{32}P]-PBt in intact cells as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at $P<0.05$ as compared to the control cells treated with basal MEM alone. **Significantly different at $P<0.05$ as compared to MβCD or HPCD treated cells.

Fig. 11. *Time-course of MβCD- and HPCD-mediated cholesterol removal modulation of TPA-induced (PKC-activated) PLD activity in vascular endothelial cells*

Confluent BPAECs (5x10^5 cells/35-mm dish) were treated for 15, 30, 60, or 120 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (0.5% wt/vol). Following cholesterol removal from cells, the treatments were removed and the cells were treated with basal MEM only or basal MEM containing TPA (25 nM) for 60 min and the PLD activity was determined as the formation of [^{32}P]-PBt in intact cells as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone. **Significantly different at P<0.05 as compared to MβCD or HPCD treated cells.

Fig. 12. *Dose-response of MβCD- and HPCD-mediated cholesterol removal modulation of DPV-induced (PTyK-activated) PLD activity in vascular endothelial cells*

Confluent BPAECs (5x10^5 cells/35-mm dish) were treated for 60 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (0.5%, 1%, or 2% wt/vol). Following cholesterol removal from cells the treatments were removed and the cells were treated with basal MEM only or basal MEM containing DPV (10 μM) for 30 min and the PLD activity was determined as the formation of [^{32}P]-PBt in intact cells as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at P<0.05 as compared to the control cells treated with basal MEM alone. **Significantly different at P<0.05 as compared to MβCD or HPCD treated cells.

Fig. 13. *Time-course of MβCD- and HPCD-mediated cholesterol removal modulation of DPV-induced (PTyK-activated) PLD activity in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated for 15, 30, 60, or 120 min with basal MEM or basal MEM containing MβCD **[A]** or HPCD **[B]** (0.5% wt/vol). Following cholesterol removal from cells the treatments were removed and the cells were treated with basal MEM only or basal MEM containing DPV (10 μM) for another 30 min and the PLD activity was determined as the formation of [^{32}P]-PBt in intact cells as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at $P<0.05$ as compared to the control cells treated with basal MEM alone. **Significantly different at $P<0.05$ as compared to MβCD or HPCD treated cells.

Fig. 14. *Modulation of oxidant (H_2O_2)-induced activation of PLD by MβCD- and HPCD-mediated cholesterol depletion in vascular endothelial cells*

Confluent BPAECs ($5x10^5$ cells/35-mm dish) were treated with basal MEM or basal MEM containing MβCD or HPCD (2% wt/vol) for 60 min. Following cholesterol removal from cells the treatments were removed and the cells were treated with basal MEM only or basal MEM containing H_2O_2 (100 μM) for 60 min and the PLD activity was determined as the formation of [^{32}P]-PBt in intact cells as described in the Methods section. Data represent mean±SD of three independent experiments. *Significantly different at $P<0.05$ as compared to the control cells treated with basal MEM alone. **Significantly different at $P<0.05$ as compared to MβCD or HPCD treated cells.

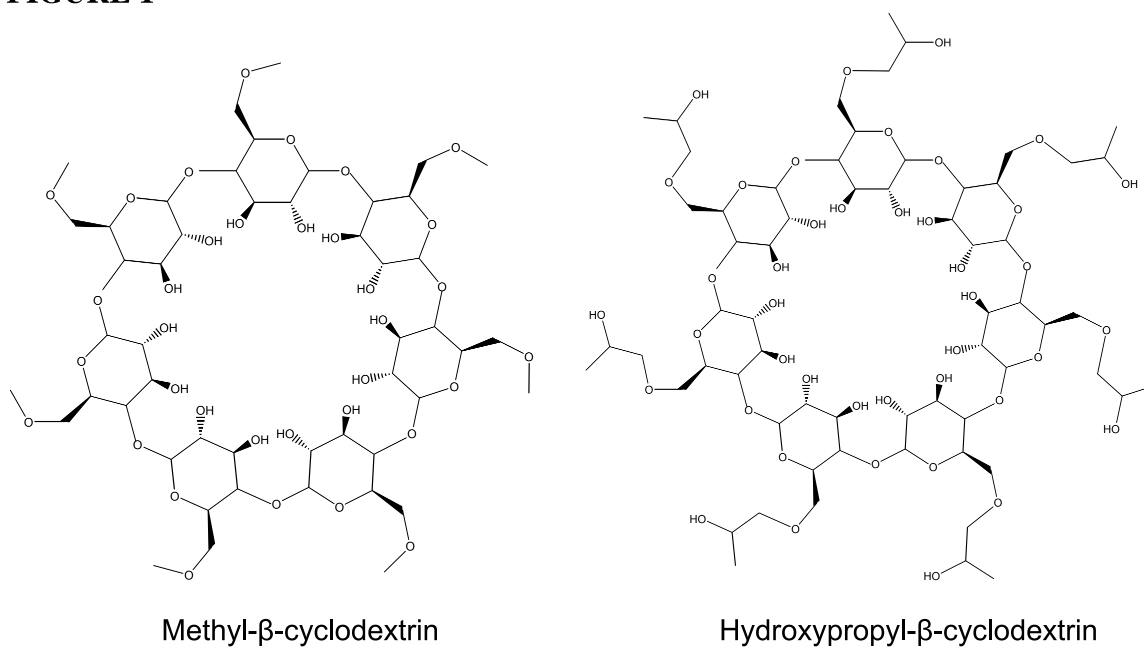
Schema-1. *Mechanism of action of PLD and generation of potent cellular lipid signal mediators (diacylglycerol, DAG; phosphatidic acid, PA; lysophosphatidic acid, LPA)*

In the presence of primary alcohol, PLD catalyzes the formation of phosphatidylalcohol (e.g. butanol is converted by PLD into phosphatidylbutanol, PBt) by transphosphatidylation, which is the basis for assaying the activity of PLD.

Schema-2. *Proposed mechanism of alterations of cell viability, function, and lipid signaling by cholesterol removal mediated by cyclodextrins*

Cyclodextrins (MβCD and HPCD) assist the removal of membrane cholesterol (also the raft-associated form), which apparently causes cytotoxicity, loss of mitochondrial function, alterations in cell morphology, and loss of cell division in vascular endothelial cells. Also, removal of cholesterol assisted by the cyclodextrins, causes modulation of protein kinases such as protein kinase C (PKC) and tyrosine kinases (PTyK), which in turn regulate the lipid signaling enzyme, phospholipase D (PLD) in the vascular endothelial cells. Oxidant-induced (e.g. H_2O_2) activation PLD is also known to be regulated by both PKC and PTyK all of which are apparently modulated by the removal of cellular cholesterol in microenvironments assisted by MβCD and HPCD.

FIGURE 1

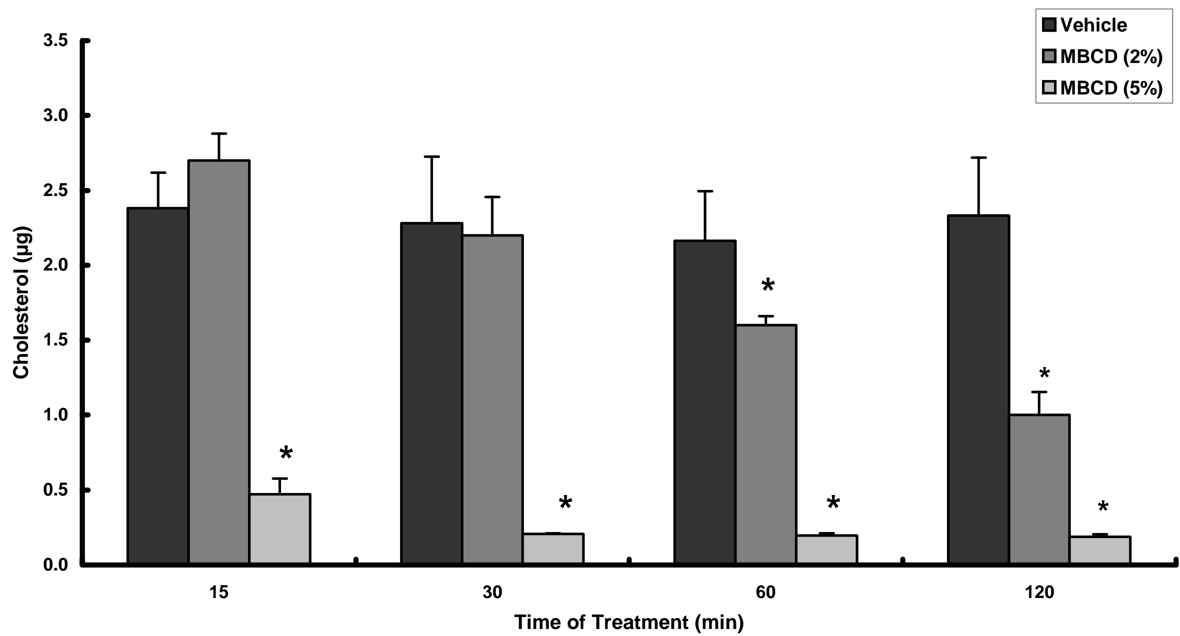


Methyl-β-cyclodextrin

Hydroxypropyl-β-cyclodextrin

FIGURE 2

A



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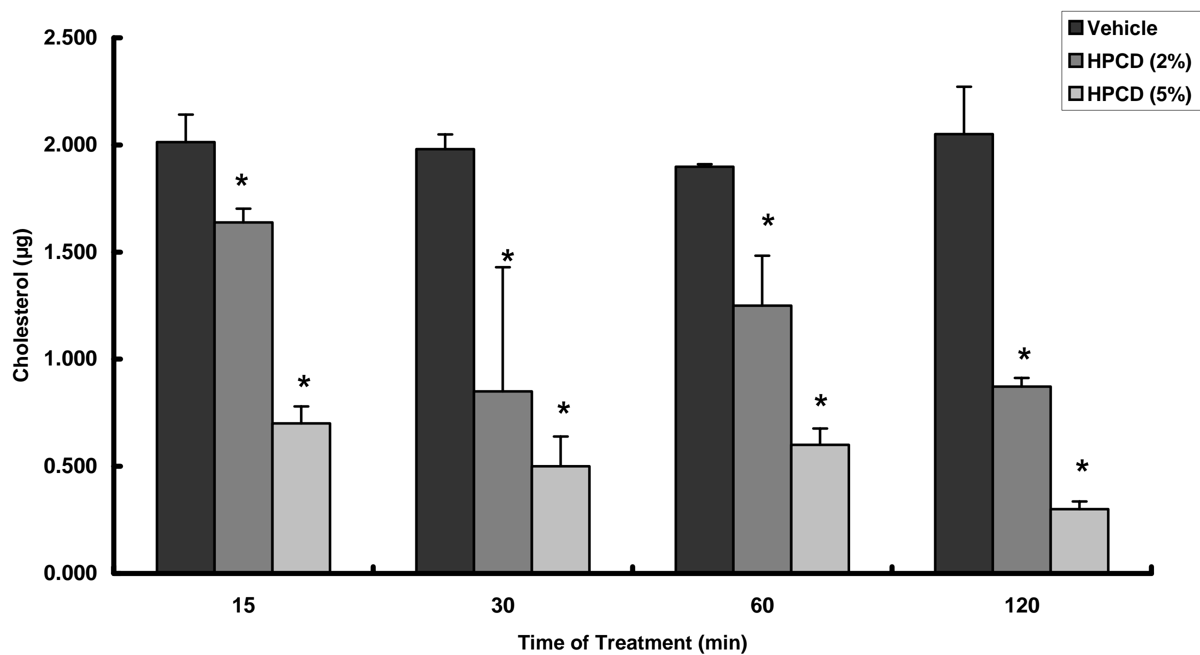

FIGURE 3

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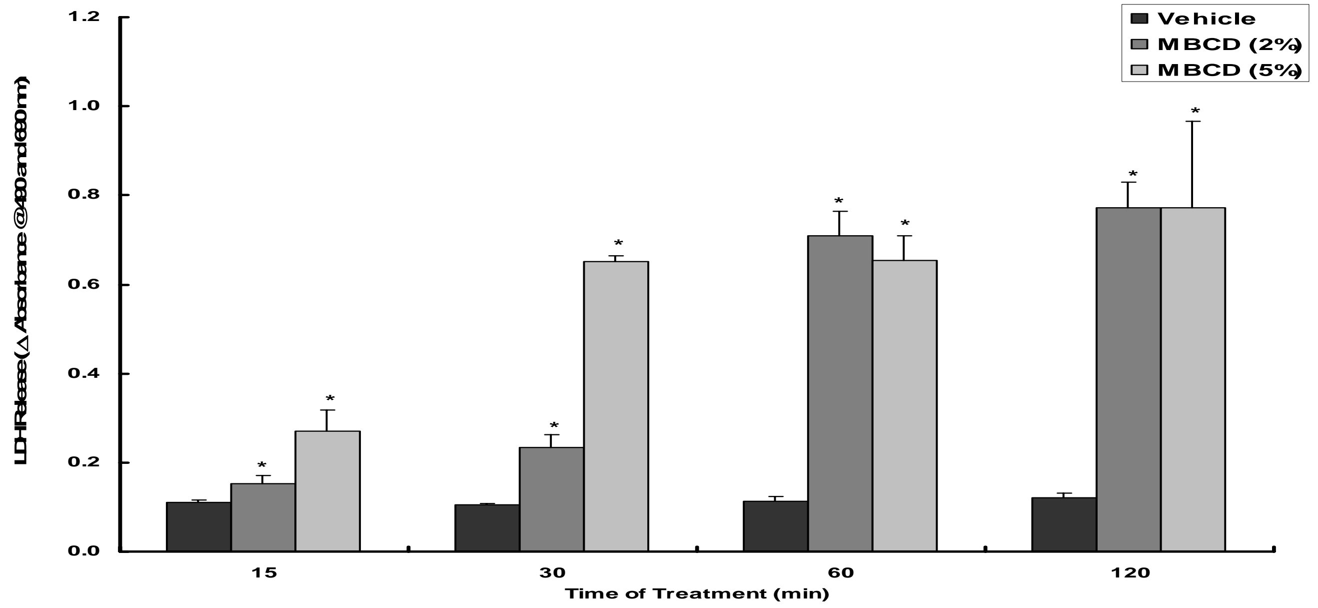

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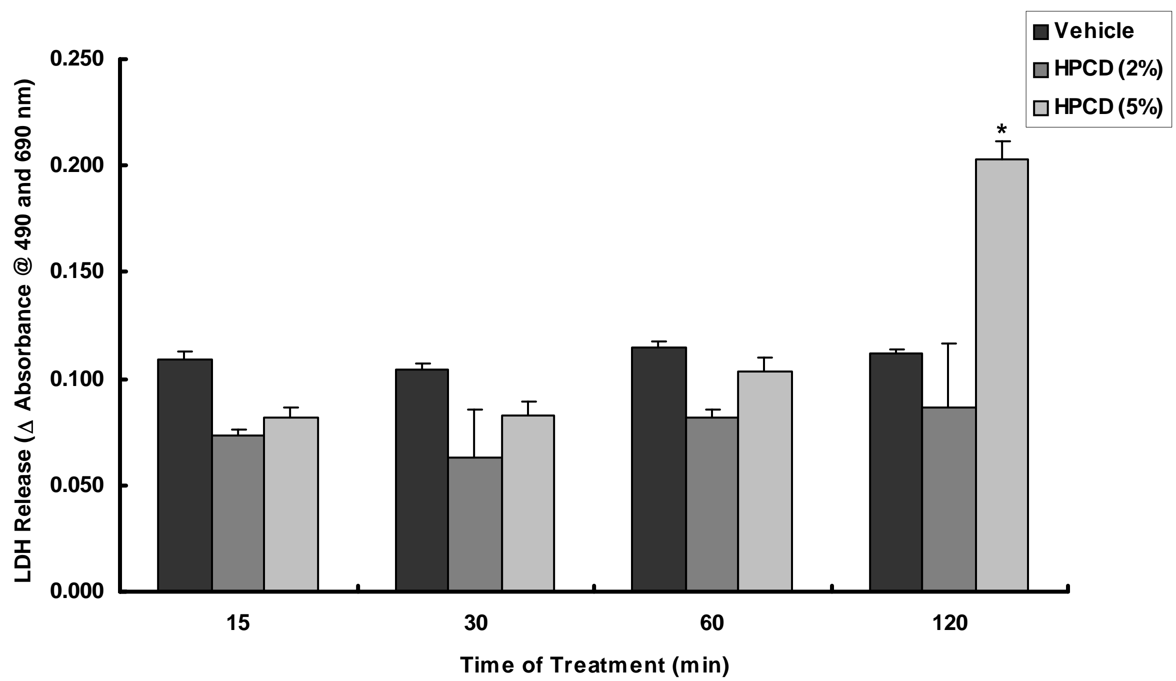

FIGURE 4

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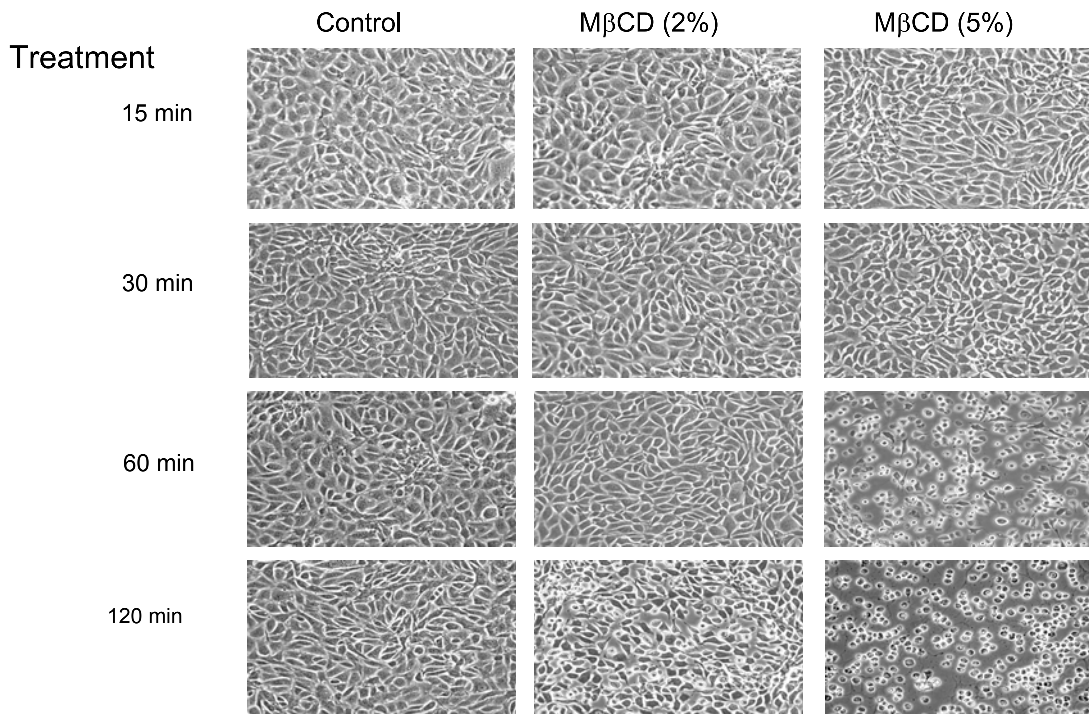

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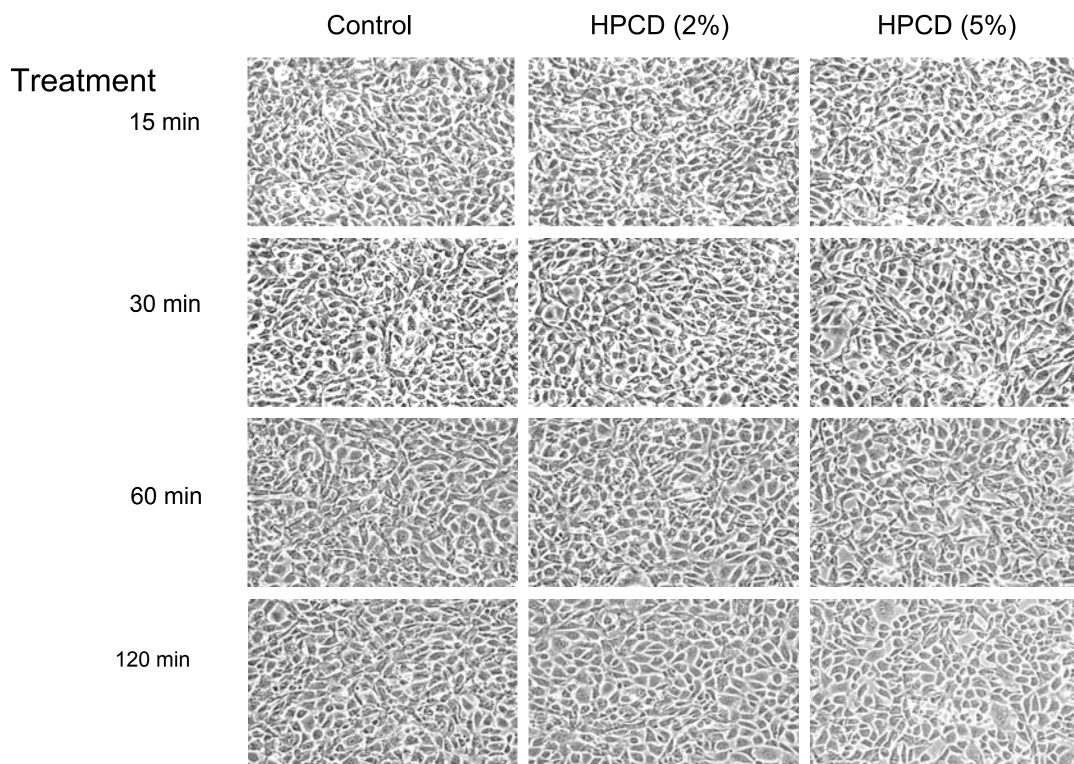


FIGURE 5

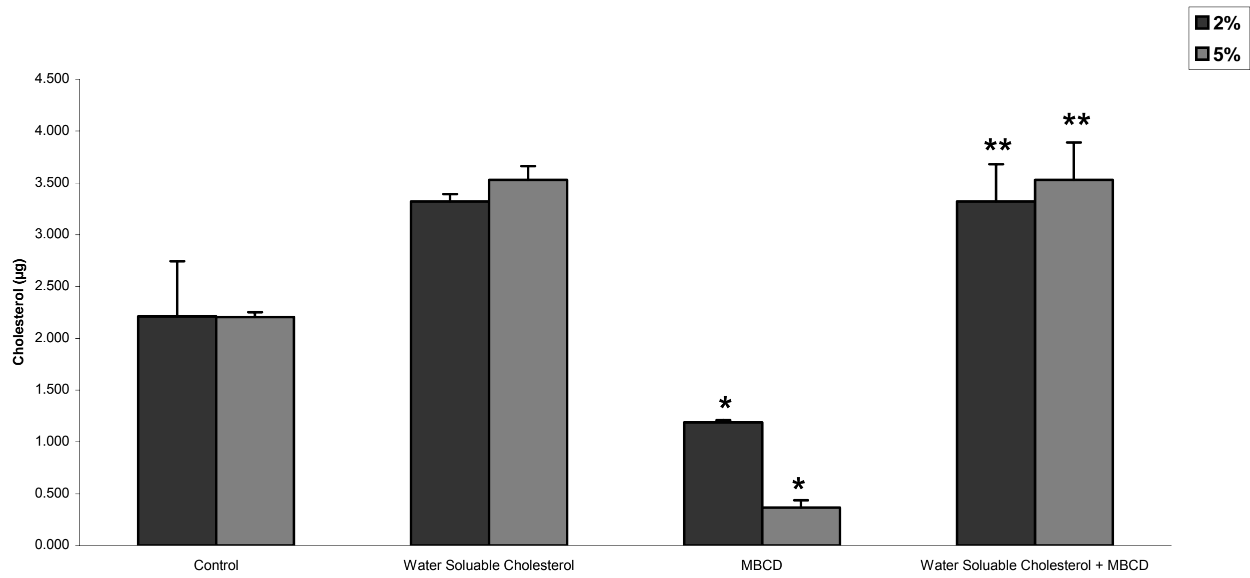


FIGURE 6

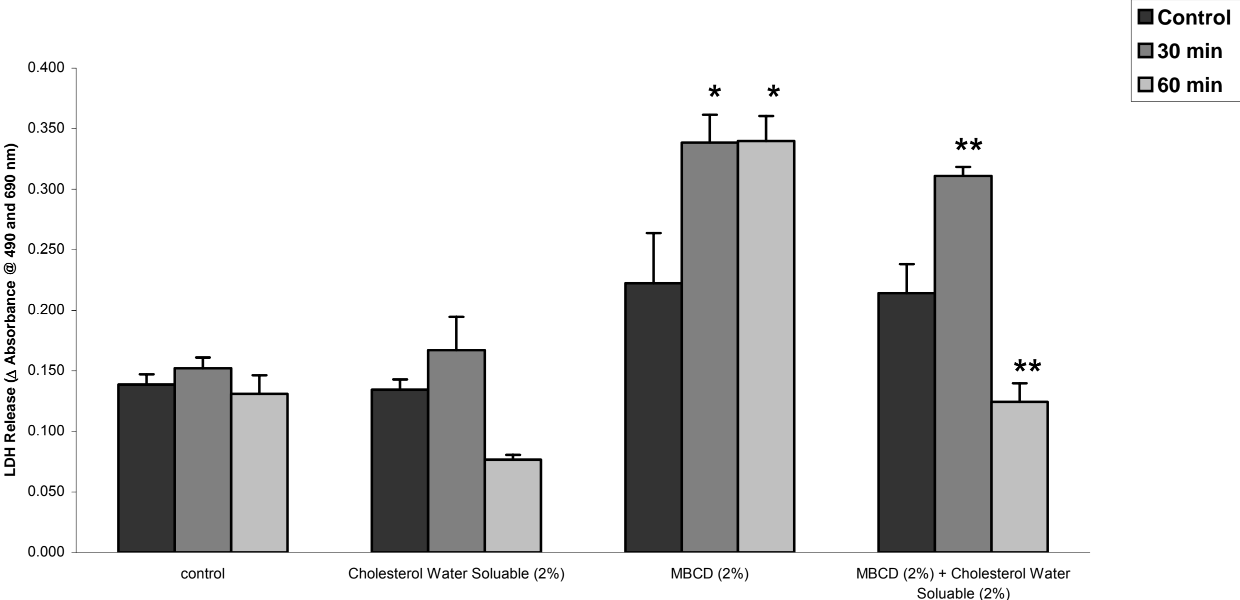
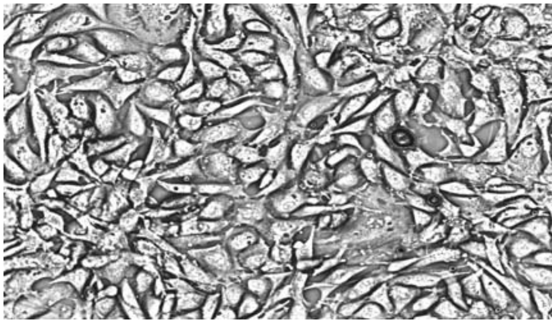
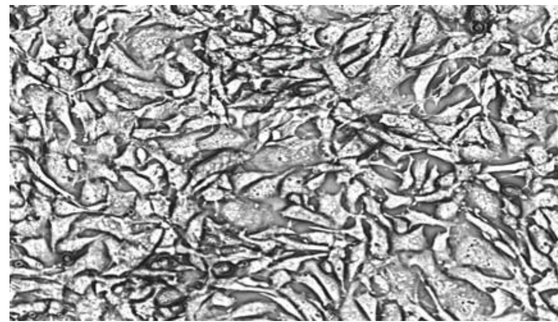


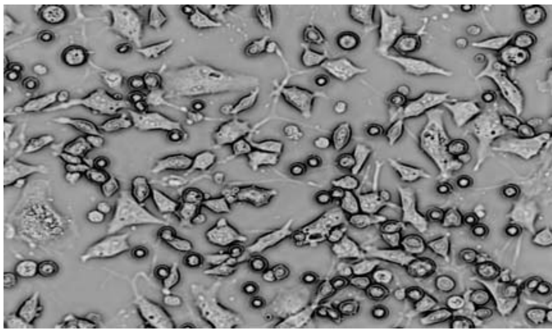
FIGURE 7



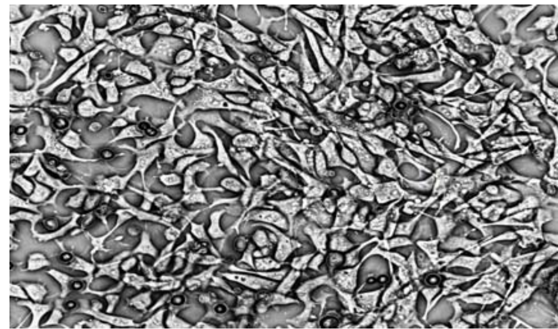
Control



Cholesterol



MβCD



Cholesterol (2%) + MβCD (2%)

FIGURE 8

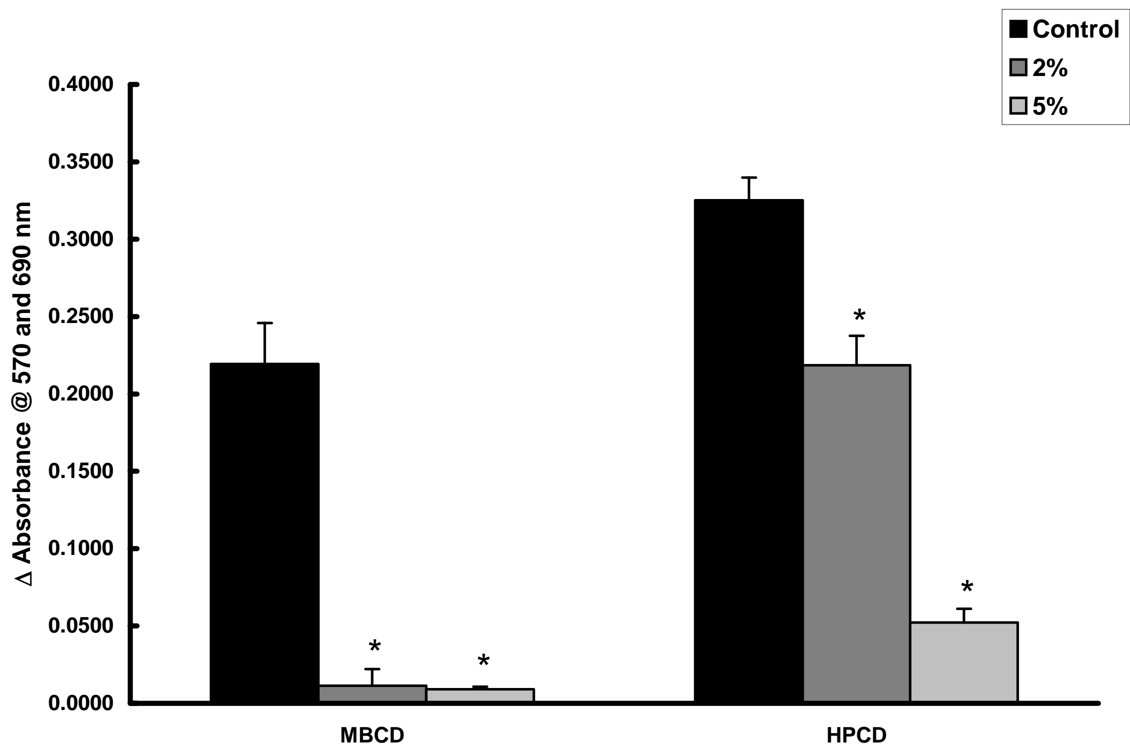


FIGURE 9

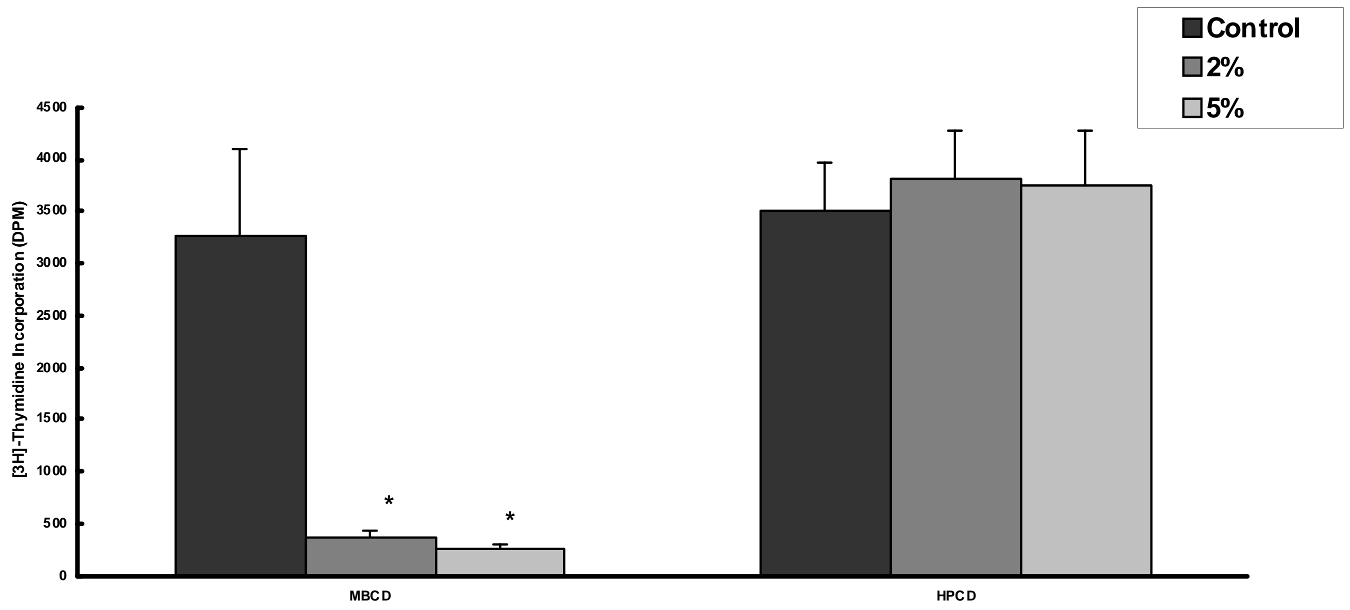


FIGURE 10

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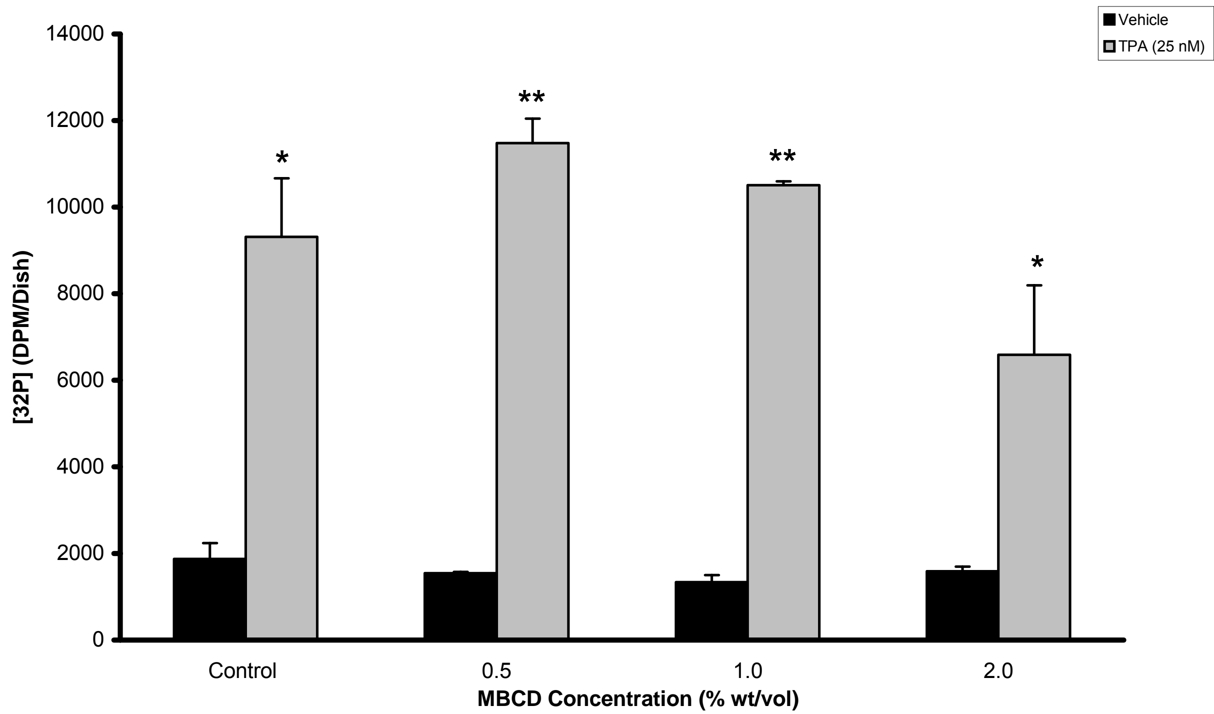

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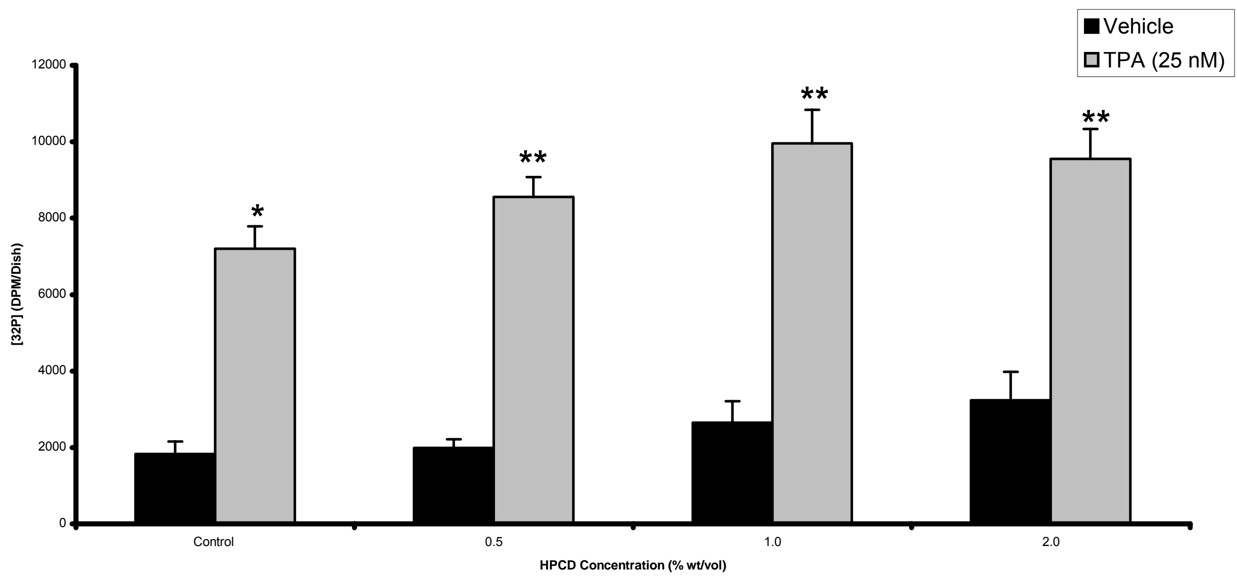

FIGURE 11

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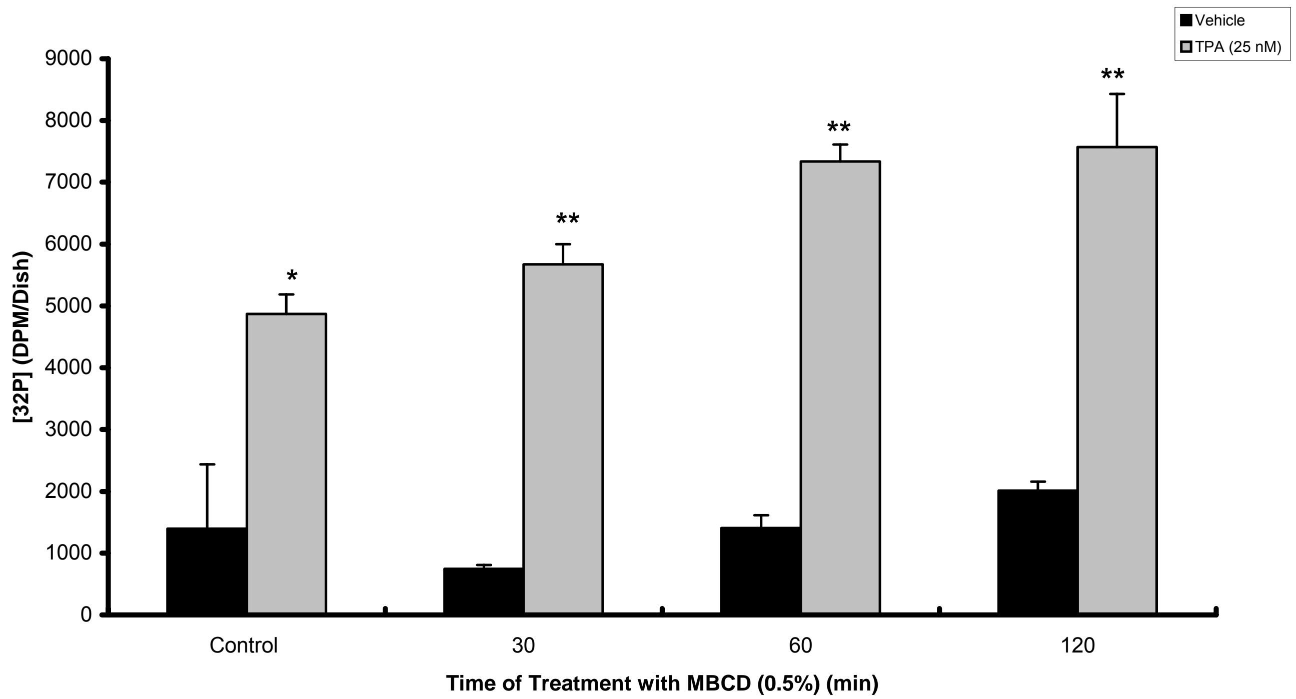

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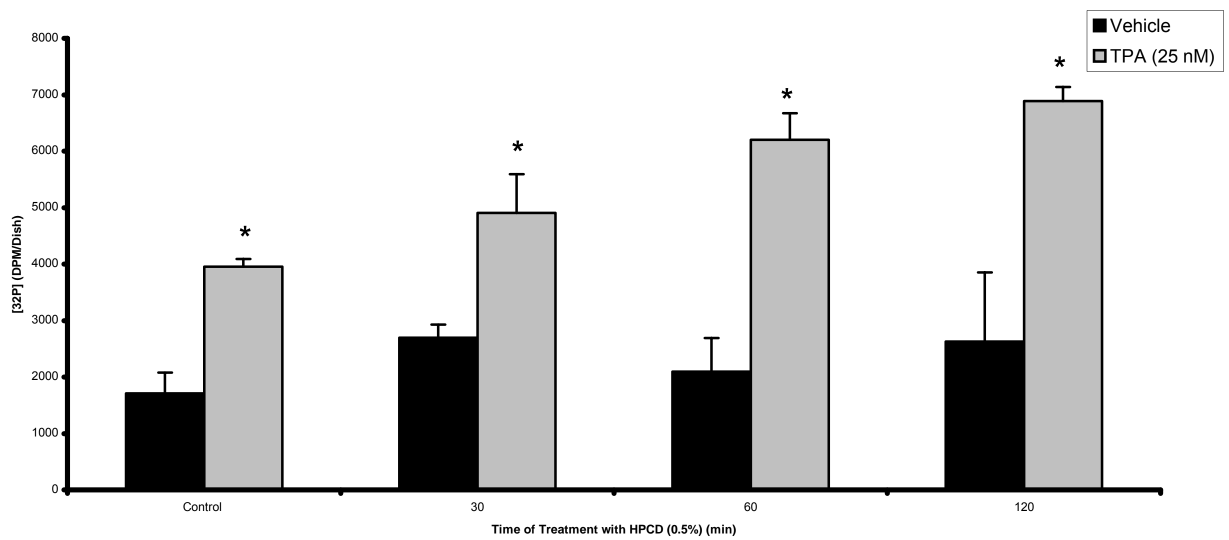
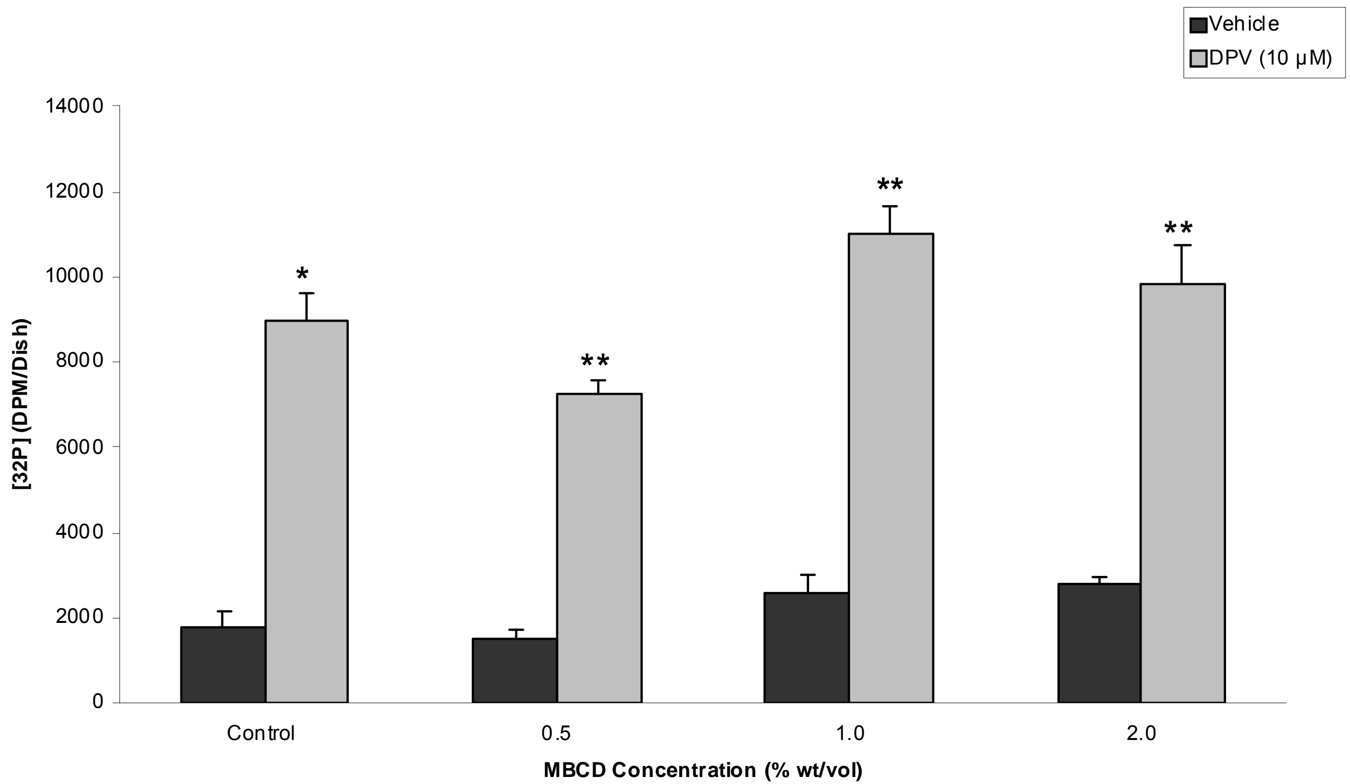


FIGURE 12

A



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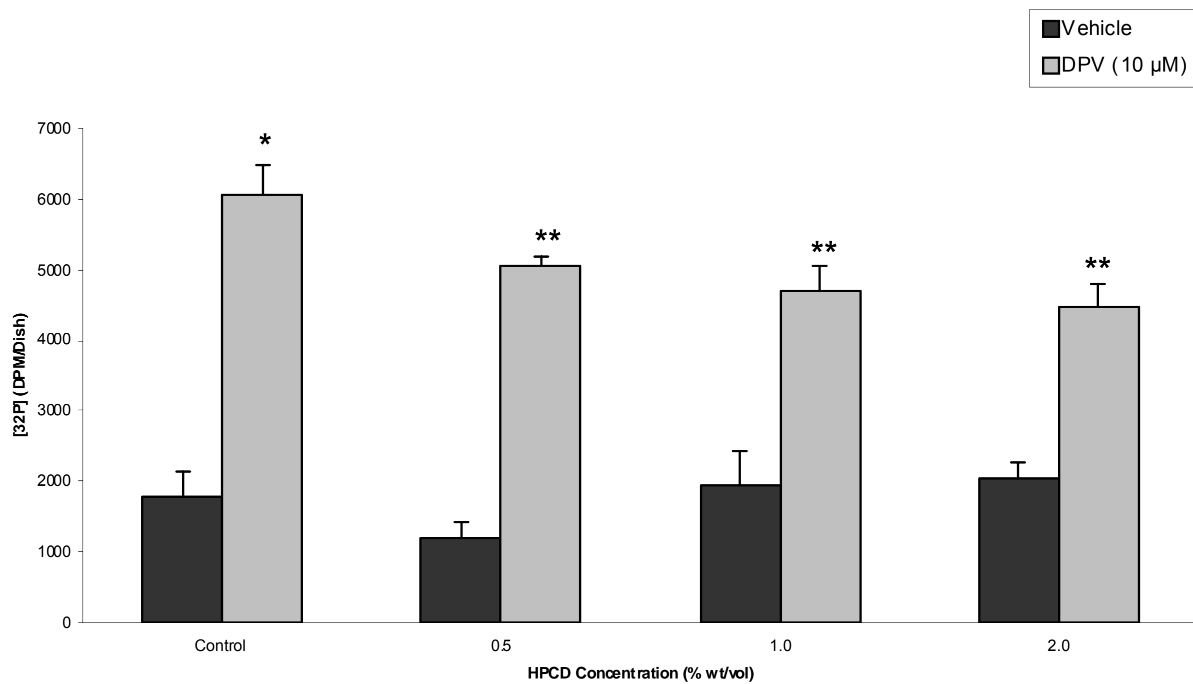
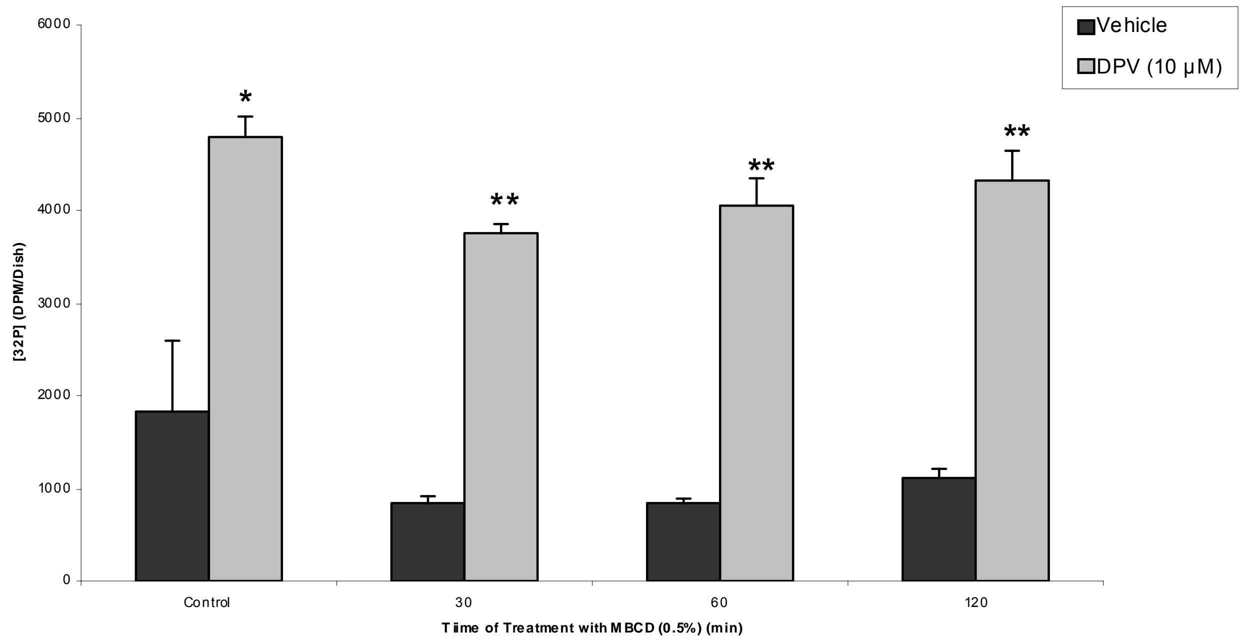


FIGURE 13

A



B

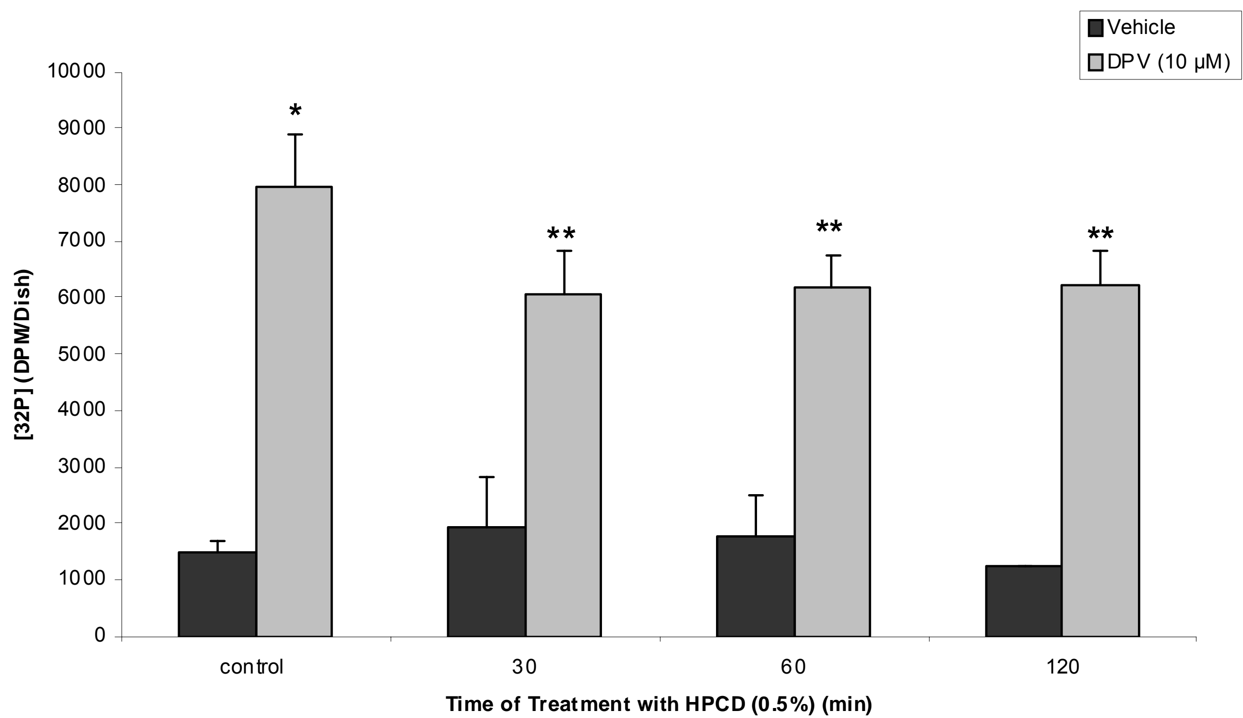
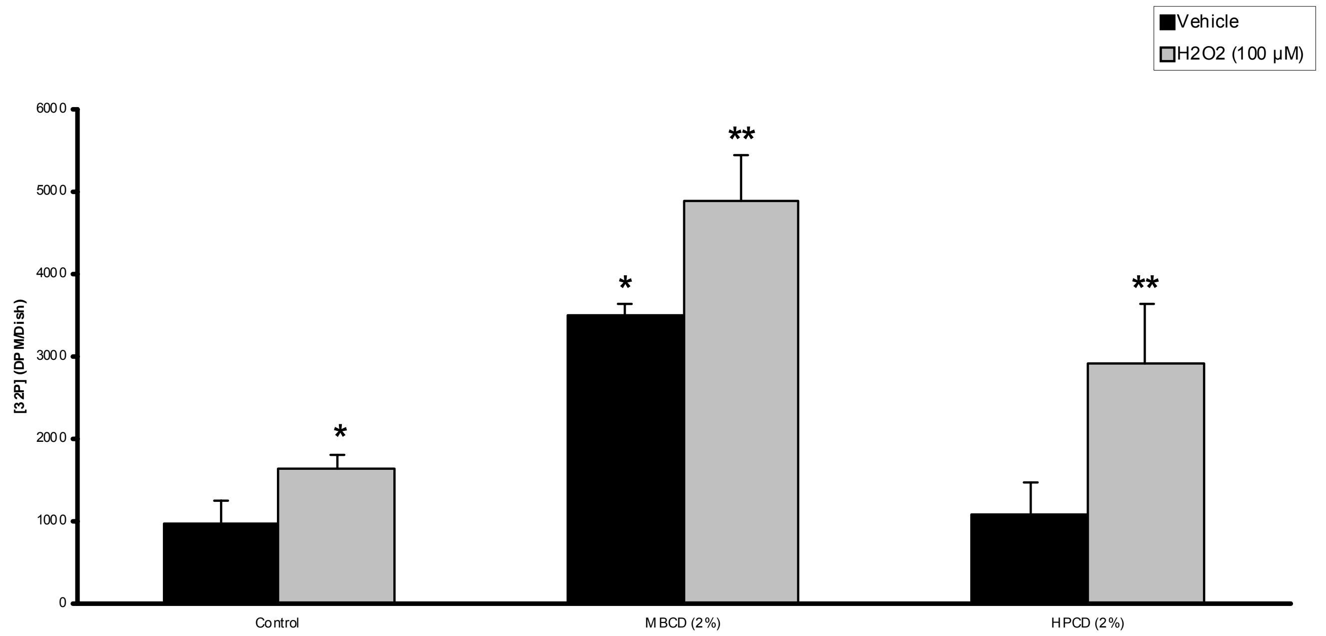
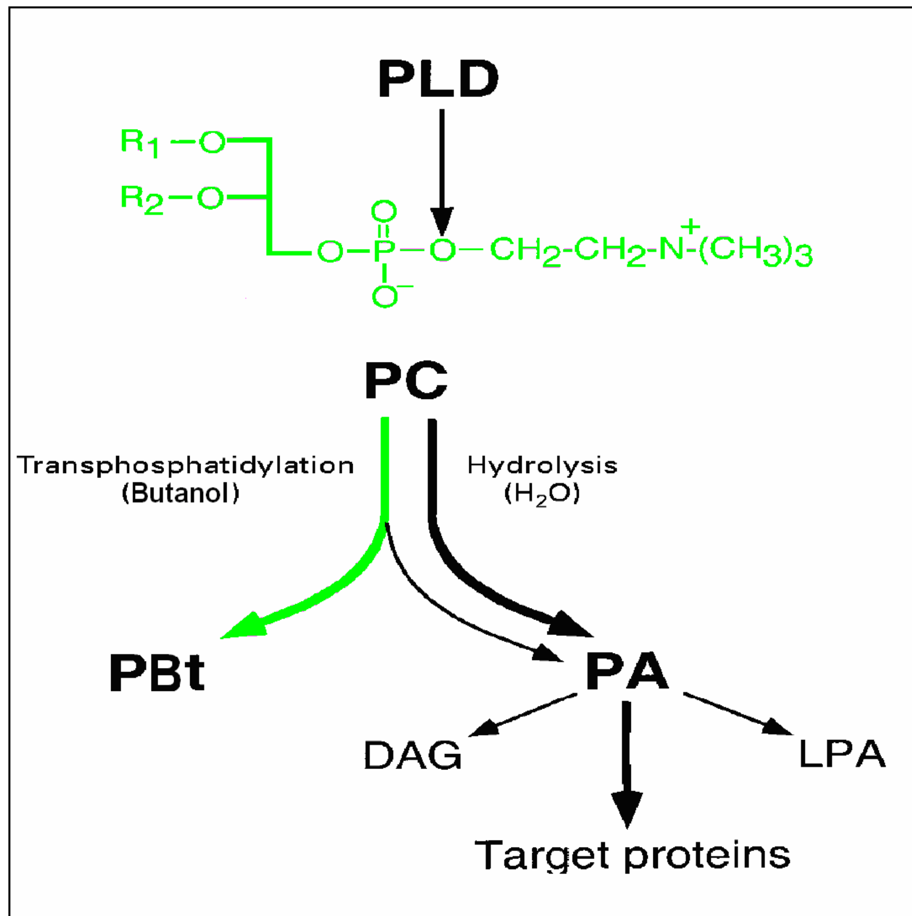


FIGURE 14



SCHEMA-1



SCHEMA-2