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Dipyridamole Reduces Oxidative Stress in Human Coronary Artery Endothelial and Hela Cell Models

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By:

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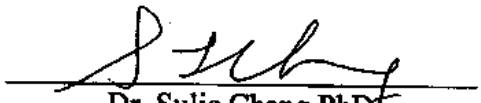

Dr. Sulie Chang PhD
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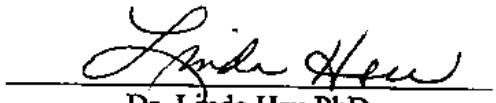

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Abstract:

Oxidative stress has been implicated in the vascular alterations that accompany diabetes and atherosclerosis. Since oxidative stress can cause the derangement of cellular metabolism, agents that suppress oxidative damage may prove useful in treating vascular disorders. In the current study, the effects of the cardiovascular agent dipyridamole, in suppressing cellular free radical production and increasing cellular viability, were examined. Overnight treatment of either human coronary artery endothelial cells (hCAEC) or human ovarian carcinoma cells (HeLa) with hydrogen peroxide resulted in significant levels of intracellular free radical production and resulted in cell death. Dipyridamole proved effective in reducing hydrogen peroxide-mediated free radical production and in increasing cell viability. Taken together, these data suggest that dipyridamole may prove useful in alleviating the endothelial cell dysfunction associated with chronic oxidative stress.

Introduction:

Chronic oxidative stress is a major causative agent in vascular endothelium dysfunction and has been implicated in vascular diseases that accompany diabetes, atherosclerosis, and some neuronal disorders, such as vascular dementia (Lum and Roebuck, 2001). Oxidative stress has been shown to alter gene expression and enzyme activities (Gerritsen and Bloor, 1993), as well as induce DNA strand breaks and suppression of protein synthesis (Jornot et al., 1991). Through the addition of oxidants such as hydrogen peroxide (H_2O_2) (Vercellotti et al., 1991) and organic peroxides (Elliott et al., 1992), it is possible to directly induce oxidative stress *in vitro*. The major contributors of oxidative damage are reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl ($\cdot OH$) and superoxide (O_2^-) radicals (Droge, 2002). ROS are derived from molecular oxygen by sequential monovalent reductions, usually occurring in the mitochondrial electron transport chain and in phagocytic vesicles (Dreher and Junod, 1995). ROS are a by-product of normal aerobic metabolism, and the cell possesses enzymatic mechanisms to balance ROS production. ROS also function as signaling molecules. For example, ROS are required for cytokine secretion, cellular defense and cell growth (Lum and Roebuck, 2001). However, at high concentrations, or in a cellular environment where antioxidant defenses are compromised, these ROS can modify lipids, proteins, and other cellular macromolecules, resulting in cell injury and/or death. ROS may also cause cellular shape alterations such as actin filament reorganization and impaired intercellular gap formation resulting in the inability to form proper cell-cell adhesions (Lum and Roebuck 2001), thereby increasing the permeability of the vascular endothelium.

Normally, during periods of tissue inflammation or injury, cellular mediators activate circulating leukocytes, and this activation allows the leukocytes to adhere to the endothelium, and ultimately penetrate the endothelial layers. Current experimental evidence suggests that the site through which this penetration occurs is through intercellular junctions (Lum and Roebuck, 2001). However, in abnormal conditions, where excessive leukocyte adhesion occurs, the increased levels of leukocyte extravasation cause tissue damage (Lum and Roebuck, 2001). Interestingly, there is a significant amount of evidence suggesting that oxidative stress promotes leukocyte adhesion to the endothelium (Lum and Roebuck, 2001). Therefore, it appears that oxidative stress has two major effects on vascular endothelium: increasing endothelium permeability and increasing leukocyte adherence, resulting in such conditions as tissue edema and leukocyte extravasation (Lum and Roebuck, 2001).

Oxidative stress can also be induced by hyperglycemic conditions (Droge, 2002). The onset of chronic hyperglycemic conditions is a direct result of the body's failure to properly manage insulin concentrations in the blood. In the body, insulin is required to lower blood glucose levels by signaling cells (particularly in fat and muscle) to take up glucose from the bloodstream. In addition, insulin inhibits hepatic glycogenolysis. The overall effect of insulin is to reduce the amount of glucose in the bloodstream; so aberrant insulin levels can lead to hyperglycemia and concomitant increase in cellular ROS.

There has been a substantial increase in the number of diabetic cases over the past two decades. This increase can be largely attributed to changes in the human environment, behavior and lifestyle (Zimmet et al., 2001). What was once considered to be a minor condition, diabetes has reached epidemic proportions worldwide, with more

than 150 million people suffering from the disease (Amos et al., 1997 and King et al., 1998). Diabetes is classified as having two main forms: Type-1 diabetes (commonly referred to as insulin-dependent diabetes mellitus (IDDM)) arises from the inability of the body to produce insulin due to the autoimmune destruction of the pancreatic islet cells. As a result, Type-1 diabetics require insulin injections throughout their lifetime (Haberman, 2000). In contrast to Type-1 diabetes, insulin injections would prove ineffective in treating Type-2 diabetes – the more common form of diabetes accounting for 90% of all diabetic cases in industrialized countries (Haberman, 2000). Type-2 diabetes (commonly referred to as non-insulin-dependent diabetes mellitus (NIDDM)) is characterized by insulin resistance and/or pancreatic insufficiency. Insulin resistance is the inability of target tissues to respond normally to insulin. Pancreatic insufficiency is defined as the inability of the pancreas to secrete enough insulin to compensate for insulin resistance. These two factors will consequently lead to an increase in blood glucose levels (Haberman, 2000).

Due to a marked increase in diabetic cases, it is imperative that therapeutic interventions be designed to alleviate the disease complications. Previous studies have targeted the lifestyle of susceptible individuals as a means to reduce the disease-associated morbidity (Brownlee, 2001). Late-stage complications of diabetes include blindness, amputations, nerve damage, and kidney disease (Porte and Schwartz, 1996). There is also increasing evidence that macrovascular complications are due to inadequate glycemic control (Brownlee, 2001). For example, diabetes has been implicated in atherosclerosis, which can prevent blood from reaching critical organs such as the brain and heart leading to stroke and coronary heart disease – the most common cause of death

among diabetics (Brownlee, 2001). Several causative mechanisms of atherosclerosis have been investigated, including dysfunctional lipid metabolism (Ginsberg 2000).

Cellular processes that produce ROS may be a plausible explanation for the increased occurrence of oxidative stress in hyperglycemic conditions. For example, the expression of various protein kinases and intercellular adhesion molecules (e.g. ICAM-1) is induced by oxidative stress allowing for increased arterial invasion by monocytes and T-lymphocytes, ultimately causing atherosclerotic lesions (Droge, 2002). There is current evidence suggesting that inflammation due to these lesions triggers a marked increase in C-reactive protein (CRP) – a molecule implicated in arteriolar plaque formation (Taubes, 2002). Additionally, it has been shown that cells subjected to hyperglycemic conditions had a defective glucose-6-phosphate dehydrogenase enzyme - the enzyme in the pentose phosphate pathway that is required for the cellular antioxidant defense system. This enzymatic defect resulted in cellular stress linked to the formation of ROS (Zhang et al., 2000). Also, the anti-atherogenic molecule nitric oxide is not produced in insulin-resistant conditions and hyperglycemia itself has been shown to inhibit production of nitric oxide in arterial endothelial cells (Brownlee, 2001). Recent studies suggest that hyperglycemia allows for an increase in the production of superoxide by the mitochondrial electron-transport chain (Droge, 2002). Overall, according to the increasing number of reports, high glucose levels may favor an enhanced production of the reactive oxygen species, including superoxide anions, hydrogen peroxide, or hydroxyl radicals (Giugliano et al., 1996).

Endothelial cells form a monolayer in every blood vessel in the circulation (Griffioen and Molema, 2000). Since they are in direct contact with the blood, they are

susceptible to various oxidizing agents circulating in the bloodstream (Lum and Roebuck, 2001). The endothelium is involved in many regulatory processes, including blood coagulation, permeability regulation, vascular remodeling (e.g. wound healing), and immune cell recruitment (Griffioen and Molema, 2000). Normally, the vascular endothelium supports cardiovascular function by promoting vasodilatation and inhibiting platelet aggregation, however these mechanisms are impaired in dysfunctional endothelium (Katusic, 2001).

Current studies suggest that the pathogenesis induced by oxidative stress begins with endothelial dysfunction (Brownlee, 2001). It has been shown that the endothelial linings of blood vessels become dysfunctional when chronically exposed to cardiovascular risk factors, including hypercholesterolemia, hyperglycemia, hypertension, and smoking (Katusic, 2001). There is an increasing amount of evidence suggesting that leukocyte adhesion to the vascular endothelium is a key component to the development of endothelial-related disorders (Morigi et al., 1998). Additionally, the activation of protein kinase C (PKC) by oxidative stressors has been shown to compromise normal endothelial cell functions including cell contraction, basement membrane production, and cell proliferation (Hempel et al., 1997). An increase in endothelium permeability also involves the actions of PKC, as well as the transcription factor NF-κB (Morigi et al., 1998). Alvarez Jr. et al. (1997) suggest that increased endothelial cell apoptosis may alter the function of the endothelium, thereby promoting atherosclerosis.

Another cell model commonly used in assessing oxidative stress is the HeLa cell. In 1951, these malignant HeLa cells were obtained from a 31-year old African-American

woman with cervical cancer (Gey et al. 1952). The ability of these cells to grow easily in culture has allowed for their usage in many types of research projects including viral replication, oxidative stress, telomerase activity, transcription factor activation, and karyotyping (Gold, 1986). In a recent study by Lee et al. (2002), apoptosis was induced in HeLa cells by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Jungas et al. (2002) studied the susceptibility of HeLa cells expressing normal and mutant cystic fibrosis transmembrane conductance regulator (CFTR) to apoptosis when exposed to H_2O_2 and found that HeLa cells with the normal CFTR are more susceptible to oxidative stress than the mutant cells. Ohashi et al. (2002) studied the effects on HeLa cells after the administration of the oxidizing molecules hemin and metalloporphyrins using the reporter molecule 2'7'-dichlorodihydrofluorescin diacetate and noted that the oxidation was inhibited by treatment of the cells with superoxide dismutase and catalase. Nunez et al. (2001) reported that oxidative damage to DNA bases in HeLa cells occurs after treatment with rhodium intercalators and photoactivation. Additionally, Kawanishi et al. (2001) report that DNA nickel compounds are capable of inducing DNA damage in HeLa cells. As a result, when considering a cell model for the current study, HeLa cells were chosen due to their proven success in past experiments involving oxidative stress.

It is apparent that endothelial cells are important for a variety of physiological and homeostatic functions. Therefore, it is imperative that their integrity be maintained. A better understanding of how oxidative stress induces cellular injury may lead to more effective therapeutic strategies to prevent tissue damage from ROS (Dreher and Junod, 1995). The cardiovascular drug dipyridamole has been implicated in preventing oxidative

stress in several cellular models, including rat E18 hippocampal neurons (Farinelli et al., 1998) and rat cortical neurons (Geller et al., 2001). Dipyridamole has a variety of pharmacological functions, including the inhibition of phosphodiesterases (Thompson, 1991) and the prevention of nucleoside uptake (Zhang and Fredholm, 1994), as well as blocking chloride transport (Garcia and Lodish, 1989) and increasing prostaglandin synthesis (Jackson et al., 1982). Iuliano et al. (1995) previously demonstrated dipyridamole's ability to inhibit lipid peroxidation and scavenge free radicals. Farinelli et al. (1998) determined that dipyridamole exerts its effects mainly through its antioxidant properties. Consequently, it is necessary to see if dipyridamole's antioxidant properties can be utilized to reduce the amount of oxidative damage suffered by endothelial cells lining the micro- and macrovasculature. It is hypothesized that oxidative stress imposed upon endothelial cells during H_2O_2 administration can be reduced with dipyridamole administration.

The current study hypothesizes that dipyridamole may prove to be a useful chemopreventative agent for the endothelium. To test this hypothesis, endothelial and HeLa cell monolayers were treated with various concentrations of hydrogen peroxide (H_2O_2) to introduce an oxidatively stressful environment. Dipyridamole was co-incubated with the H_2O_2 to determine if this drug had the ability to prevent the cellular death caused by the oxidizing conditions.

Methods:

Cell Culture:

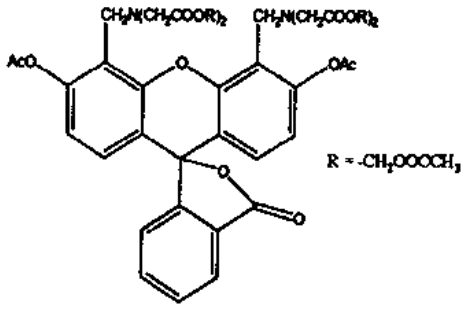
Human coronary artery endothelial cells (hCAEC) and all hCAEC tissue culture reagents were obtained from Clonetics, BioWhittaker (Walkersville, MD) and kept in a –80°C freezer until use. The cells were thawed rapidly and then maintained in T-75 culture flasks with Endothelial Cell Basal Medium (EBM; +5% FBS, 3mg/ml bovine brain extract). The cells were passaged when the cell monolayer reached 80% confluence. Cell monolayers were rinsed with 5ml Hepes buffered saline solution (HBSS). The cells were dislodged using 5ml of 0.05% w/v trypsin in 0.53 mM EDTA for 3 minutes and then neutralized with 5ml trypsin neutralizing solution. For experiments, approximately 1×10^4 cells per well were plated into 2 24-well culture dishes in a 37°C humidified incubator at 5% CO_2 : 95% air. All sterile cell culture plastic dishes and pipettes were obtained from Corning (Corning, NY). The cells were maintained in a 37°C humidified incubator at 5% CO_2:95% air.

HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and kept in a –80°C freezer until use. All HeLa cell tissue culture reagents were obtained from InVitrogen Life Technologies (Grand Island, NY). The cells were thawed rapidly and routinely maintained in T-75 culture flasks with Dulbecco's Modified Eagle Medium (DMEM; 5% FBS/100 units ml^{-1}, penicillin/100μg ml^{-1}, 2 mM L-glutamine.) The cells were passaged at 80% confluence. When passaging the cells, 5ml calcium/phosphate-free Dulbecco's phosphate buffered saline (DPBS) was used to rinse the cells. The cells were dislodged using 5ml of 0.05% w/v trypsin in 0.53 mM EDTA for 3 minutes and then neutralized with 5ml DMEM containing 5% FCS. For experiments,

approximately 2 x 10^4 cells per well were plated into 2 24-well culture dishes in a 37°C humidified incubator at 5% CO_2 : 95% air.

Determination of Calcein-AM uptake and retention:

Calcein-AM is a useful probe of cell viability because it does not fluoresce extracellularly and is freely permeable to cell membranes. Once inside the cell, the intracellular esterase enzymes hydrolyze this compound into a fluorescent product with an excitation wavelength at 485 nm and an emission wavelength of 530 nm. Interestingly, once this probe is cleaved, it has superior cellular retention properties due to its newly formed charged side-chains (Molecular Probes) (Figure 1a,b).



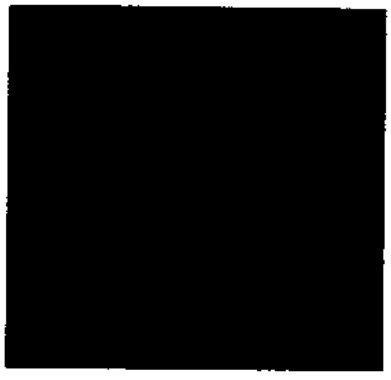


Figure 1: (a) Structure of calcein-AM fluorescent probe (Molecular Probes) (b) calcein-AM fluorescence as seen in cultured rat neuronal population (Blake AD, unpublished)

hCAEC monolayers in 24-well culture dishes were washed with phosphate buffered saline solution (1ml $well^{-1}$) and treated with increasing concentrations (0.10 μM, 0.25 μM, 1.0 μM, and 2.5 μM) of calcein-AM (Molecular Probes, Eugene, OR) in phenol red-free EBM (0.5ml $well^{-1}$) over a 100-minute time-course. Calcein fluorescence was measured using a Cytofluor-4000 fluorescent plate reader (Applied Biosystems, Foster City, CA) programmed to perform fluorescence readings every 5 minutes for 100 minutes

at a set photomultiplier gain of 70.

In order to determine if the hCAECs were capable of retaining the fluorescence over the desired time course, hCAECs plated on 24-well culture dishes were incubated with 1.0μM calcein in phenol red-free EBM over 5-minute intervals at 37°C. After each interval, the cells were washed with DPBS. The fluorescence was measured immediately after washing to determine if the cells were capable of maintaining the initial fluorescence. The same procedure was used for the HeLa cell model.

Detection of Free-Radical Production using CM-H_2DCFDA Fluorescence:

hCAEC monolayers in 24-well culture dishes were washed with DPBS (1ml $well^{-1}$) and treated in the presence or absence of increasing concentrations (0.03%, 0.3%, and 3%) of H_2O_2 (Fisher Scientific, Fair Lawn, NJ) (6 wells per concentration). Parallel control wells received no treatment. The cells were then incubated for 30 minutes at 37°C. After the incubation period, 3 of the 6 treated wells were additionally treated with 0.5ml 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes, Eugene, OR) in phenol red-free EBM (5 μM final concentration) and incubated for 10 minutes. This compound passively diffuses into cells where intracellular esterases cleave the acetate groups, effectively trapping the molecule within. Meanwhile, the chloromethyl portion of the molecule can react with glutathione and other intracellular thiol-containing proteins allowing for subsequent oxidation by free radicals. The fluorescent product forms after contact with these intracellular free radicals resulting in a fluorescent signal that is retained inside the cell. The excitation wavelength for this compound is 485 nm and the emission wavelength is 530 nm (Figure 2).

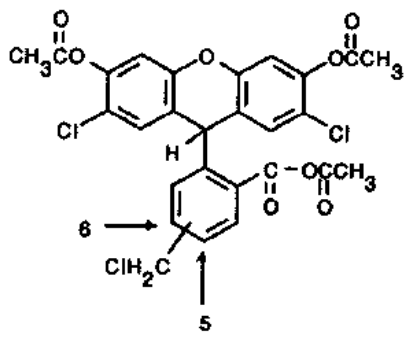


Figure 2: Structure of 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA)

After the 10-minute incubation period, the cells were rinsed with DPBS to remove any residual CM-H_2DCFDA from the wells. The retained monolayer fluorescence was measured using a fluorescent plate reader at a photomultiplier gain of 70 over a 45-minute time period.

Drug application (hCAEC):

hCAECs plated on 24-welled culture dishes were treated with 3% H_2O_2 (1ml $well^{-1}$) simultaneously with either the drug dipyridamole (10 μM) or glutathione (1 mM) (Sigma, St. Louis, MO) for 30 minutes at 37°C (1ml $well^{-1}$). All treatment solutions were prepared in EBM. After the 10-minute incubation period, the treatment solutions were aspirated and the cells washed with DPBS (1ml $well^{-1}$). 0.5 ml CM-H_2DCFDA (5 μM) was then added to each well. The cells were returned to the 37°C incubator for 10 minutes. After the 10-minute incubation period, the EBM was aspirated and the cell-associated fluorescence was measured for 30 seconds using a fluorescent plate reader.

H_2O_2 Toxicity on HeLa cells:

HeLa cell monolayers in 24-well culture dishes were incubated at 37°C for 24 hours with decreasing concentrations of H_2O_2 (3% to 0.0003%) in DMEM. After the 24-hour incubation period, the cells were rinsed with saline (-calcium/-phosphate) and treated with 1.0 μM calcein-AM in saline (0.5 ml $well^{-1}$) for 20 minutes at 37°C. Following the 20-minute incubation period, the calcein-AM solution was aspirated and the cells were rinsed with saline and measured for fluorescence.

Dipyridamole application (HeLa cells):

HeLa cell monolayers in 24-well culture dishes were treated with either 10 μM dipyridamole (1ml $well^{-1}$) alone, 0.005% H_2O_2 alone (1ml $well^{-1}$), or a mixture of 1ml Dipyridamole and 1ml 0.005% H_2O_2 (combined volume of 2ml $well^{-1}$) for 24 hours at 37°C. After the 24-hour incubation period, the treatment solutions were aspirated, rinsed with saline, and treated with 1 μM calcein-AM in saline for 20 minutes at 37°C. After the 20-minute incubation period, the calcein-AM was aspirated, rinsed with saline and the cells were measured for fluorescence.

Data analysis and determination of statistical significance:

The obtained fluorescent data were analyzed using GraphPad Prism 3™ software (GraphPad, San Diego, CA). Statistical significance was determined using one-way analysis of variance ($p < 0.05$ representing significant difference) and a Newman-Keuls multiple comparison test.

Results:

Determination of calcein-AM concentration and exposure time

In order to use calcein-AM as a fluorescent indicator for cell viability, it is essential to use a large enough concentration that will produce measurable results when read by the fluorescent plate reader. However, it is equally important to use a concentration that it not toxic to our cell culture. In order to determine a suitable concentration, a human coronary artery endothelial cell population was subjected to a range of concentrations of the fluorescent indicator over 10-minute intervals. According to the data obtained (Figure 3), calcein-AM concentrations of 2.5μM and 1.0μM produced robust signals, whereas lower concentrations (0.25μM and 0.10μM) did not. In deciding between 2.5μM and 1.0μM, 1.0μM produced a linear curve that increased gradually over the time course. Conversely, a concentration 2.5μM produced readings that increased sharply and eventually leveled off, resulting in a curve with varying slopes. Due to the inconsistency of these results when using the 2.5μM concentration, the 1.0μM concentration was chosen for use in viability determinations.

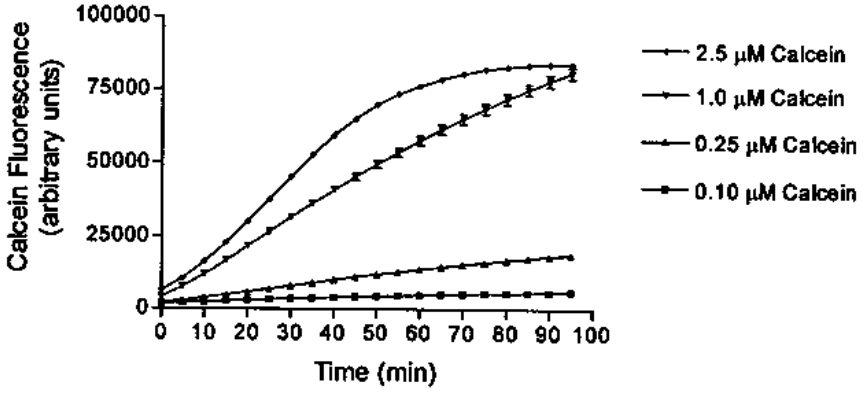


Figure 3: Time-dependent calcein uptake by hCAEC

After choosing the concentration of the viability indicator it was necessary to chose an exposure time. According to the graph in Figure 3, it was determined that an exposure time of 20 minutes will yield the most consistent results since this is the time in which the 1.0μM curve is at its maximal linearity.

Calcein-AM retention

After determining a suitable calcein-AM concentration for detecting cell viability, it is necessary to ensure that the cells are capable of retaining the fluorescence that is produced within the cell. To accomplish this, the endothelial cells were exposed to the target calcein-AM concentration (1.0μM) over a specific time period and then immediately rinsed with buffered saline solution. According to the results obtained (Figure 4), the cells were capable of retaining the fluorescence after rinsing since there was very little reduction of fluorescence following rinsing.

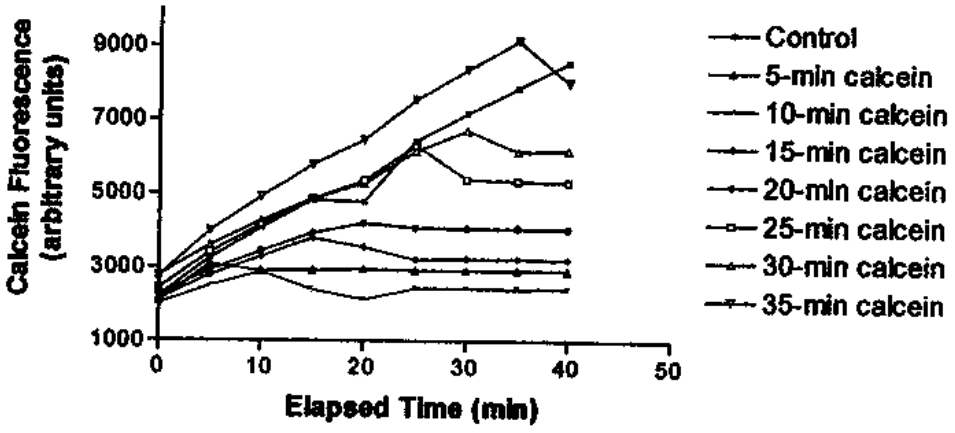


Figure 4: Time-course of intracellular calcein retention

H_2O_2-induced free-radical production is detected using CM-H_2DCFDA

In order to determine the presence of free-radical production by endothelial cells during H_2O_2 exposure *in vitro*, the free-radical-detecting compound CM-H_2DCFDA was utilized. A cell monolayer was exposed to a 3% H_2O_2 concentration, while another monolayer was exposed to a 0.3% H_2O_2 concentration. Immediately following H_2O_2 exposure, CM-H_2DCFDA was used to detect free-radical production. According to Figure 5, there is an indication that a larger H_2O_2 concentration leads to an increase in free-radical production as detected by CM-H_2DCFDA fluorescence. Although these results are not significant, there appears to be sufficient evidence that free-radical production has, in fact, increased during H_2O_2 exposure.

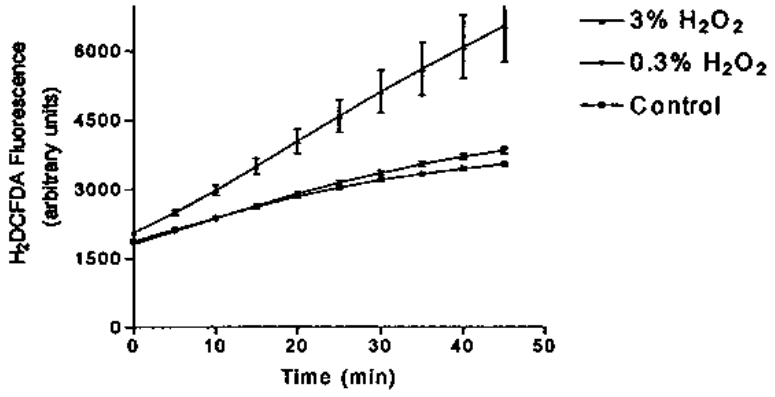


Figure 5: Time-course of hydrogen peroxide-induced free radical production in hCAEC

Chemosuppression of free-radical production:

To determine whether the drug dipyridamole is capable of suppressing free-radical production in human coronary artery endothelial cells during periods of oxidative stress, its effects were compared to those of a well-known free-radical inhibitor - glutathione.

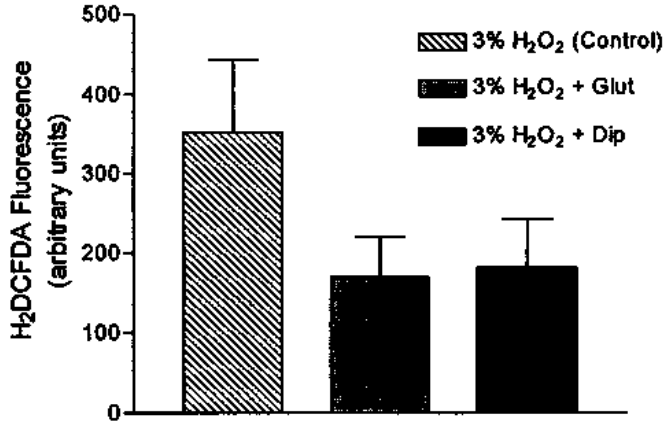


Figure 6: Dipyridamole and glutathione suppress free radical production in hCAEC

According to the data depicted in Figure 6, dipyridamole appears to have similar free-radical inhibition capability to that of glutathione. Although these results are not statistically significant, they may suggest a possible therapeutic role for administration of exogenous dipyridamole, since its effects seem to mimic those of endogenous glutathione in this particular trial.

Determination of the EC_{50} on the HeLa cell model

In order to observe the preventive effects of dipyridamole during an oxidatively stressful period, it was first necessary to determine a concentration of H_2O_2 that would destroy 50% of the HeLa cell monolayer population. Varying concentrations of H_2O_2 were tested several times and a dose-response curve was generated using GraphPad Prism software (Figure 7).

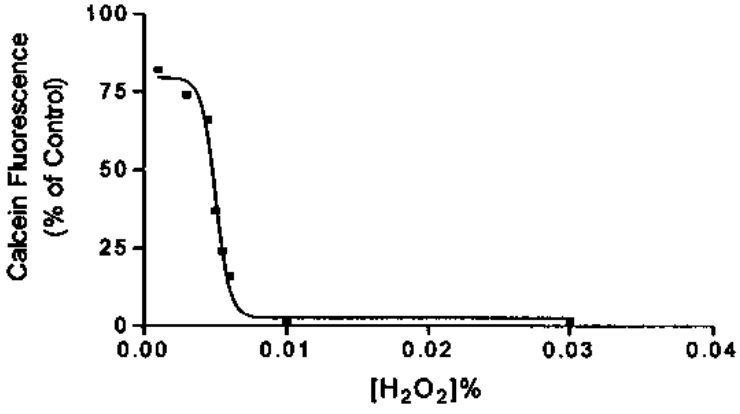


Figure 7: Hydrogen peroxide reduces HeLa cell viability in a concentration-dependent manner

From Figure 7 it was determined that a concentration of 0.005% H_2O_2 was the EC_{50}. As a result, 0.005% H_2O_2 in combination with dipyridamole will be used in the subsequent experiment to test dipyridamole's ability to prevent the damage done at this H_2O_2 concentration.

Dipyridamole prevents cell death during oxidatively stressful conditions in HeLa model

HeLa cells that were exposed to the EC_{50} H_2O_2 concentration (0.005%) for 24 hours were completely destroyed. These data were significantly different than the control (untreated) values. Dipyridamole was co-incubated with 0.005% H_2O_2 for 24 hours and

appeared to have a preventative mechanism. There was approximately a two-fold increase in the number of viable cells (significantly different) that were exposed to H_2O_2 plus dipyridamole as opposed to H_2O_2 alone. These data are summarized in Figure 8.

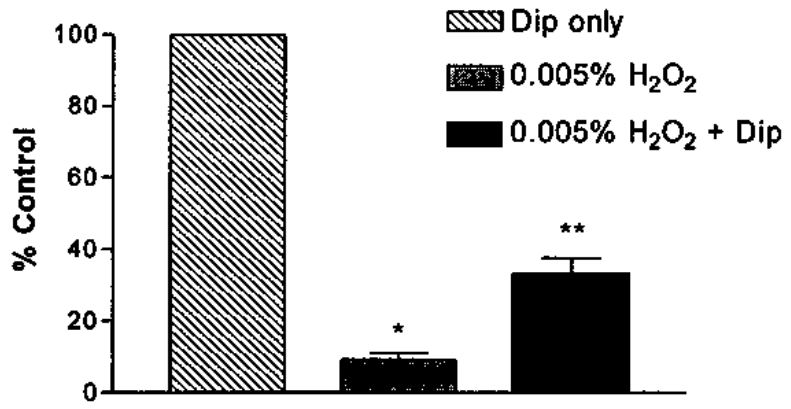


Figure 8: Dipyridamole reduces hydrogen peroxide induced cell death in HeLa cells

Discussion:

This study demonstrated that the clinically useful cardiovascular drug dipyridamole has anti-oxidant properties that can suppress oxidative stressors. This is an important finding since oxidative damage is a leading cause of endothelial dysfunction in diseases such as diabetes and atherosclerosis. In understanding the effects of oxidative stress in the body, endothelial cells were chosen as the principal model because these cells line the macrovasculature and are susceptible to oxidative stress (Lum and Roebuck, 2001). In the present study, oxidative stress was induced using various concentrations of H_2O_2, a potent free radical generator. The end-point of the study was indicated by cell death. In order to determine which cells were viable after oxidative treatments, the fluorescent probe calcein-AM was utilized.

In choosing an optimal concentration for the calcein-AM probe, it was important to minimize cellular toxicity while optimizing the signal-to-noise ratio. If excessive amounts of the probe were administered, the physiological characteristics of the cell would be altered, possibly producing spurious results. After preliminary experiments, a final extracellular concentration of 1μM was utilized since it provided an optimal signal-to-noise ratio and allowed experiments to be conducted in an expedient manner.

Calcein-AM is known to have a high cellular retention ability in established cell lines, however, since the coronary artery endothelial cells used in this experiment were a primary culture, it was necessary to determine if calcein-AM was retained in these cells as well. The results in Figure 4 clearly depict the monolayer's ability to retain the probe since the fluorescence intensity remains relatively constant after rinsing. This was an important finding since it proved that (1) the human coronary artery endothelial cells

were able to take up the probe in a time and concentration-dependent manner and (2) the probe was retained within the intracellular compartment. As such, the calcein-based viability assay proved to be a highly reproducible measure of hCAEC viability.

In attempting to prove the experimental hypothesis, the first step was to induce oxidative stress in the hCAEC cells. In order to do this, a strong oxidizing compound (H_2O_2) was used. Consequently, it was necessary to evaluate the levels of intracellular oxidative stress. To measure the levels of intracellular free radical production, the reporter molecule 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was chosen.

To determine whether CM-H_2DCFDA would provide a reliable measure of intracellular oxidative stress in hCAEC, cells were labeled with a 1μM concentration and incubated with two different concentrations of H_2O_2 (3% and 0.3%). These concentrations are very high, relative to physiological conditions, and were used solely for the purpose of detecting a robust signal. Figure 5 shows that an increase in CM-H_2DCFDA fluorescence was achieved with both concentrations of H_2O_2 and this increase was significantly higher than background. These results demonstrate that H_2O_2 can cause free radical production within hCAEC, which can be reliably detected by measuring the intracellular fluorescence. Based upon the ability of CM-H_2DCFDA to measure free radical production, the effects of dipyridamole on intracellular oxidation were compared to those of glutathione, an endogenous cellular free radical scavenging tripeptide.

Although the results in Figure 6 are not statistically significant, there is an indication that an oxidizing situation was present, which was subsequently reduced using glutathione and dipyridamole. Although Figure 6 depicts that H_2O_2 effects were not

completely inhibited by either antioxidant, concentration-dependent dosing of dipyridamole and glutathione might provide an approximate effective concentration (EC_{50}) value for future experimentation.

The second step in examining the experimental hypothesis involved establishing the EC_{50} at which half the viability of our cell population was compromised. This EC_{50} of H_2O_2 would be more comparable to physiological conditions and would allow for a direct determination of dipyridamole's effects. For this portion of the experiment, the HeLa cell model was utilized since this model has been extensively used in defining the molecular events that accompany oxidative stress. Using data acquired from several trials, Figure 7 shows a concentration-dependent response that was modeled using a four-parameter logistic curve fit (GraphPad Prism 3™) resulting in an overall EC_{50} of 0.005% H_2O_2. The concentration-response curve exhibited a steep slope and the EC_{50} fell within a very narrow range given that concentrations above the EC_{50} were extremely toxic, while concentrations below the EC_{50} were less effective.

Finally, the chemoprotective effects of dipyridamole were examined on HeLa cells. The EC_{50} concentration of H_2O_2 was used in combination with 10μM dipyridamole (Figure 8). Dipyridamole clearly reduced H_2O_2-induced toxicity in the HeLa cell cultures, effectively reducing the toxicity of H_2O_2 by 50%. Approximately 35% of the cell culture (as compared to the control) survived, in the presence of 10μM dipyridamole, compared to 15% viability in the absence of the drug. These results provide the first evidence, using both a normal cell line (hCAEC) and a transformed cell line (HeLa), that dipyridamole has a chemoprotective effect against oxidative stressors. Overall, these data clearly indicate that dipyridamole may play a protective role during periods of oxidative stress.

Conclusion:

In this study, it has been demonstrated that, similar to glutathione, the drug dipyridamole has anti-oxidant properties in the human coronary artery endothelial and HeLa cell models. This finding is significant since oxidative damage is a leading cause of morbidity and mortality among individuals suffering from diseases such as diabetes and/or atherosclerosis. Further research must be performed in order to investigate the possible therapeutic applications for dipyridamole as a treatment for diseases related to oxidative stress. Once the pharmacokinetics of dipyridamole can be determined, the administration of the drug into patients who are at risk for oxidative damage may prove to be an invaluable tool in the treatment of diseases such as diabetes and atherosclerosis.

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