White Paper on In-Vitro Bioactivity of ASEA™ Related to Toxicity, Glutathione Peroxidase, Superoxide Dismutase Efficacy and Related Transcription Factors

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Description:

This is a technical paper describing a variety of preliminary results from in vitro experiments, performed at national research institutions, investigating the bioactivity of a certain redox signaling compound, ASEA™, when placed in direct physical contact with living cells. Specific investigations include in vitro toxicity and antioxidant efficiencies of the master antioxidants glutathione peroxidase (GPx) and Superoxide Dismutase (SOD) inside living cells and the translocation of two well-studied transcription factors (NF-kB, NRF2) known to regulate toxic response and antioxidant production in human cells. Some preliminary work on concentration dependence was also done as well as cell proliferation, counts associated with induced oxidative stress in human cells.

Objectives of Investigations:

The objectives of the investigations were (1) to determine if any signs of toxicity (NF-kB activation) are manifest when varying concentrations of a certain redox signaling compound, ASEA™, are placed in physical contact with living cells, (2) to determine if such direct contact affects the antioxidant efficacy of glutathione peroxidase (GPx) and superoxide dismutase (SOD) and (3) to determine if such contact activates translocational transcription (NRF2) associated with increased expression of antioxidants in living human endothelial cells and to verify the expression of such transcription factors by Western Blot analysis, (4) to determine the effect of this redox signaling compound on proliferation cell counts of human cells and associated markers (LDH) for cell viability and health, (5) to determine the effects of this redox signaling compound on cells that were stressed with cytokines (Cachexin), radiation and serum starvation.

Theory Overview

The immune-supporting Redox Signaling supplement, ASEA™, contains a redox-balanced mixture of Redox Signaling molecules [both reactive oxygen species (ROS) and reduced species (RS)] that are involved in a

large variety of pathways and receptor-site activity in human cells. For example, when cells are damaged, for any reason (ex. toxins, DNA breaks or infections), the native Redox Signaling messengers inside the cells can become imbalanced, most often manifest by the accumulation of intracellular oxidants and ROS (oxidative stress). The cell, so affected, will activate defense and repair mechanisms aimed to restore proper redox-signaling homeostasis and proper cellular function. If repair efforts are unsuccessful and normal homeostatic redox balance is not able to be restored, then within a few hours, the excess oxidants and ROS in such cells will facilitate apoptotic processes to internally digest and destroy the dysfunctional cell. Healthy neighboring cells will then divide to replace it. A complete field of science called "redox signaling" has been founded to study such processes, with literally thousands of references available.

It is the nature of certain redox signaling molecules, when unbalanced or isolated, to elicit immediate recognizable toxic responses in exposed living cells; hydrogen peroxide is one example of such a redox signaling molecule. The first-line cellular response to toxic substances involves the translocation of NF-kB into the nucleus as a precursor to the inflammatory response and other defense mechanisms. The movement of NF-kB into the nucleus can be visibly tracked in a living cell under a fluorescence microscope with the aid of fluorescent tag molecules. The observation of nuclear translocation of NF-kB is a sure marker that a toxic response has been initiated. Even low-level toxicity is detectable with this catch-all method; low-level concentrations of hydrogen peroxide, for example, produce an easily distinguishable positive toxic response.

A separate transcription factor, NRF2, moves into the nucleus in response to low-level oxidative stress and facilitates the increased production of antioxidants. Again, by the use of fluorescent tags, the nuclear translocation of NRF2 can be seen in cells under a fluorescence microscope. NRF2 nuclear translocation is a second-line-of-defense mechanism known to increase the production of protective enzymes and antioxidants such as glutathione peroxidase and superoxide dismutase. NRF2 translocation will often accompany low-level NF-kB activation and NF-kB activation (almost) always precedes NRF2 translocation. Substances that exhibit low-level toxicity, such as trace homeopathic toxins, have long been used to activate the NRF2 pathway in order to stimulate these natural defend-repair-replace mechanisms.

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Enzymatic efficacy of antioxidants, such as Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD), can be determined through standardized ELISA tests that measure the time-related reduction of certain oxidants introduced into cell lysates after the living cells have been exposed to the test substance for a given period of time. The reagents of the ELISA test must be chosen as not to interfere or interact with the test substance. Other critical factors such as the time of exposure and concentration dependence must be experimentally determined.

Western Blot methods also exist to experimentally determine the quantities of GPx or SOD in cell lysates. These well-established molecular separation techniques and can be used to directly verify whether the quantity of such antioxidant enzymes has been increased in the sample. Measured antioxidant efficiency, however, remains the best indication of cellular antioxidant defense.

Monitoring cellular proliferation, cell counts and chemical indicators of cellular death are also commonly used to determine cellular viability and gross response to stressors such as radiation, cytokines and toxins. Cachexin, for example, is a potent toxin, a cytokine, that elicits immediate toxic responses and build-up of oxidative stress in exposed cells. Cells, so stressed, exhibit a greater tendency to undergo apoptosis and die, thereby releasing internal proteins (such as LDH) into the surrounding serum.

Normally, when the introduction of such stressors and toxins elicit oxidative stress conditions in the cell cultures, cell counts will fall, cellular proliferation will subside, and serum LDH levels will rise, indicating that cell death is occurring in the culture. Hydrogen peroxide, radiation and serum starvation can also elicit similar responses. Redox signaling messengers, as outlined above, are intimately involved in cellular reception of and response to such stressors; redox messengers are involved in mediating antioxidant production and action to protect the cells, repair mechanisms necessary to fix DNA and structural damage and also in mediating the apoptotic process that results in cell death.

Increasing the concentration of such redox messengers in the serum may serve to augment the efficiency of these normal cellular processes. The exact action of various redox signaling mixtures must be determined experimentally. Independent unpublished studies, involving Mass Spectroscopy, Florescent Spectroscopy and Electron Spin

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Resonance, have unmistakably verified the existence of several kinds redox signaling molecules in the immune-supporting supplement, ASEA™. Well-established redox electrochemistry also validates the existence of such redox signaling molecules. The stability of this redox-balanced mixture is many orders of magnitude greater than expected. The confirmed preservation of unstable moieties in this supplement might be explained by the existence of certain stable molecular complexes, some of them verified by mass spectroscopy, that can shield radical interactions. Intellectual property agreements, however, prevent the disclosure of the details.

Experimental Methods:

The following research was conducted on a best efforts basis by a senior researcher at a national laboratory and is designed to assess basic mode-of-action when the redox signaling, ASEA™, is placed into direct contact with human cells:

1. The initial dose range projected for in vitro studies was extrapolated from a 10 ml ASEA/kg equivalent oral dose from human trials.

2. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) ELISAs were used to determine whether ASEA alters enzymatic activity in murine epidermal (JB6) cells.

3. LDH (non-specific cellular death) levels and cell proliferation rates were determined for various cell types exposed to ASEA.

4. Human microvascular endothelial lung cells (HMVEC-L) were treated with ASEA and cell lysates were analyzed by GSH-Px and SOD ELISAs to determine whether antioxidant enzyme activities are altered.

5. HMVEC-L cells were treated with a phosphate buffered saline solution (PBS)negative control, 5% and 20% concentrations of ASEA and a Cachexin positive control to determine the nuclear translocation activity of the p65 subunit of NF-kB (cytokine transcription) at 30, 60, 90 and 120 min. intervals. Fluorescent microscopy techniques were employed to image cellular response.

6. Step (4) was repeated except nuclear translocation activity of P-Jun was determined as an extension/verification of step 4.

7. Two cultures of HMVEC-L cells, one with normal random cell cycles and another with serum starvation were treated with low < 1% concentrations of ASEA to determine the nuclear activity of NRF2 (antioxidant transcription) at 30, 60, 90 and 120 minute intervals compared to a negative (PBS) control.

8. A Western Blot analysis was done on extra-nuclear and intra-nuclear fractions, separated by differential centrifugation, of serum starved HMVEC-L cell cultures exposed to < 1% ASEA compared with a positive hydrogen peroxide control to determine phosphorylation events (oxidant action) in the extra-nuclear fraction and NRF2 (antioxidant transcription) in the intra-nuclear fraction at 0, 30, 60, 90 and 120 min. intervals.

9. Normal random cell phases of HMVEC-L cells were exposed to radiation and then treated with ASEA. Cell counts were taken to determine survival.

10. The efficacy of Cachexin reception in confluent-phase and normal-phase HMVEC-L cells was determined through changes in extracellular and intracellular LDH activity in cells exposed to various mixtures of Cachexin, PBS and ASEA solutions.

Objective 1: Determine In Vitro Toxicity

Experimental Methods used to Assess Toxic Response in Primary Human Lung Microvascular Endothelial Cells (HMVEC-L):

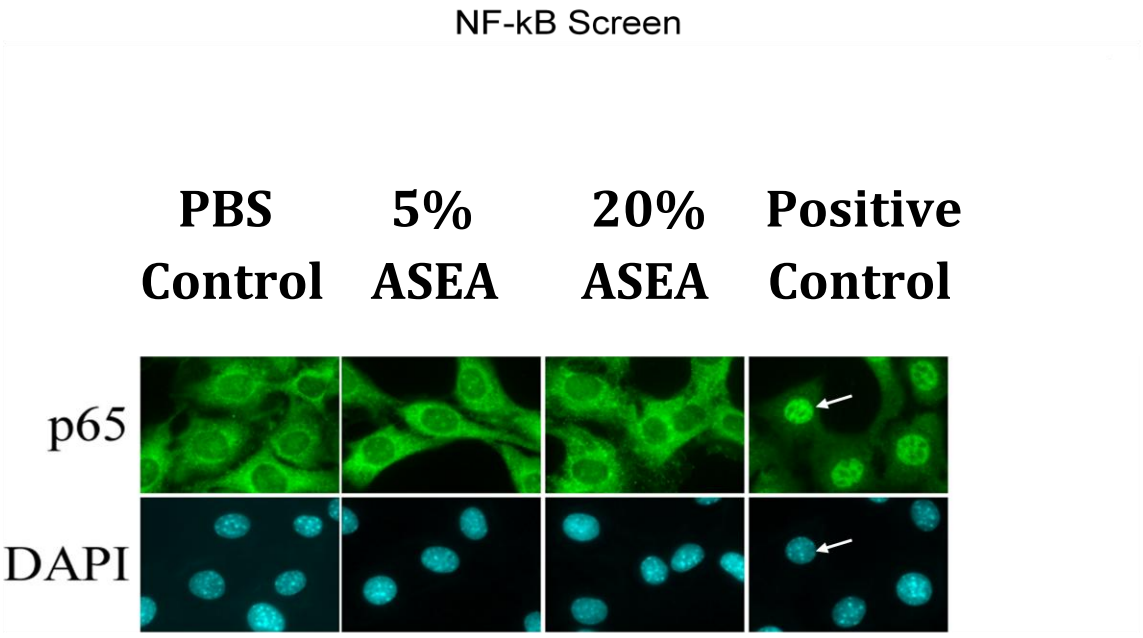
HMVEC-L cells (catalog # CC-2527) were purchased from Lonza (Walkersville, MD) as cryopreserved cells (Lot# 7F4273). Cells were thawed and maintained according to manufacturer's directions. Cell culture medium (proprietary formulation provided by Lonza) contained epidermal growth factor, hydrocortisone, GA-1000, fetal bovine serum, vasoactive endothelial growth factor, basic fibroblast growth factor, insulin growth factor-1 and ascorbic acid.

HMVEC-L Cell cultures in normal random cell cycles were exposed to high-concentration ASEA in the serum medium, concentrations of 5% and 20%, and analyzed in conjunction with cultures exposed to phosphate buffered saline solution (PBS) as non-toxic negative control and Cachexin (5 ng/ml) as a positive control (highly toxic). At intervals of 0, 30, 60, 90, and 120 minutes, aliquots of cells from each culture were placed under a fluorescent microscope, stained by

fluorescent dyes designed to tag the p65 subunit of NF-kB along with a DAPI fluorescent nuclear stain that aids the computer software to find the nuclei. Computer automated imaging techniques were used to determine the relative degree of translocation NF-kB into the nucleus via fluorescent analysis over several cells. As a reminder to the reader, P65 NF-kB translocation is the first-phase non-specific cellular response to toxicity. Thus the movement of the NF-kB into the nucleus, as seen visually in the microscope images, is a sensitive indicator of general toxic response.

Results of HMVEC-L Cells p65 subunit NF-kB screen for toxicity:

Typical cell images are shown below for each culture. Translocation of p65 subunit of NF-kB into the nucleus was not seen in any cell cultures exposed to high-concentration ASEA. Automated analysis confirmed this and indicated no toxic response at 0, 30, 90 and 120 minutes. In contrast, Cachexin exposed cells exhibited an immediate sustained toxic response.



Cachexin is positive control and induces the translocation of p65 subunit of NF-kB from cytosol into nucleus. DAPI staining shows position of nuclei in these images (see white arrow). ASEA (5 and 20% final v/v) did not induce nuclear translocation of NF-kB at 30, 60 and 120 min time points.

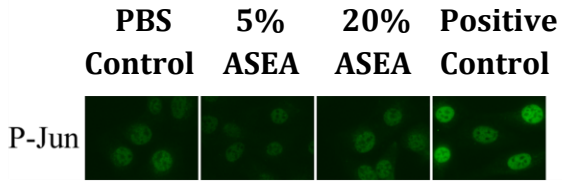
Given this null indication of toxicity after exposure to high concentrations of ASEA, another test was performed to confirm behavior.

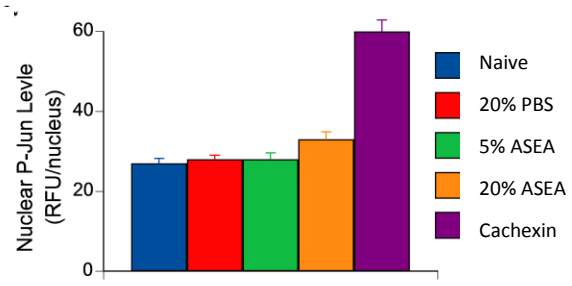
Additional Method to Assess Toxic Response of HMVEC-L Cells (P-Jun):

A similar methodology as that employed with NF-kB was employed to determine the nuclear translocation of an anti-phospho-Jun (AP-1 P-Jun) antibody index (P-Jun is another toxicity-related redox-responsive transcription factor). HMVEC-L cells were again exposed to high-concentration ASEA. All procedures were similar to the NF-kB analysis except for the substitution of P-Jun fluorescent indicators and automated measurements taken over 100 cells in order to increase sensitivity. An additional naïve (untouched) culture was also analyzed.

Results for P-Jun screen for toxicity:

AP-1 screen (redox responsive transcription factor)





AP-1 index determined using anti-phospho-Jun (P-Jun) antibody. AP-1 is nuclear localized and upon activation, the phosphorylation status of P-Jun is increased. Anti-P-Jun antibody binds to the phosphorylated form reflected as an increase in fluorescence intensity (see Cachexin control). A consistent trend reflecting an increase in P-Jun levels was not observed for cells treated with 5% or 20% ASEA at 30, 60 and 120 min time points, while the Cachexin positive control significantly increased nuclear P-Jun levels at 30 min.

Again no toxic response was observed; there was no significant accumulation of P-Jun in the nuclei of cell cultures exposed to high concentrations of ASEA. Automated analysis indicated no toxic response at 0, 30, 90 and 120 minutes, with a slight but non-significant increase for 20% ASEA at the 30 minute time point; at other time points no increase was detected. In contrast, the Cachexin exposed cells (positive control), as expected exhibited an immediate sustained toxic response.

Conclusions and Discussion on Results for Primary HMVEC-L Cell Toxicity:

The results of the P-Jun analysis concurred with the response seen in the NF-kB analysis. For both tests, there was no significant difference between ASEA exposure and that of the negative PBS control for healthy random-phase HMVEC-L cells. This confirmed lack of toxicity was somewhat unexpected for this mixture of redox signaling molecules, considering that some of them, if isolated from the mixture, are known to elicit an immediate response.

Since nuclear translocation of NF-kB and P-Jun are typically the first responders to serum toxicity and are known to initiate the inflammatory response, especially in the ultra-sensitive human endothelial cells, healthy human cells when directly exposed to ASEA, are not expected to exhibit defensive behavior nor initiate inflammatory processes (such as the release of inflammatory cytokines). It is not certain from this data whether exposure would suppress or reverse the inflammatory process.

Blood serum levels of such redox signaling molecules, for all in vivo oral applications, would not exceed serum concentrations of 1% and typically would be less than 0.1%. Serum levels are expected to drop over time due to enzymatic breakdown of the components. Independent in vivo pharmacokinetic studies indicate that the active components in ASEA have approximately a 17 minute half-life in the blood and thus would be effectively cleared from the blood within a few hours. Thus no toxic response is expected due to exposure of healthy human cells at such levels. It has been seen in these in vitro studies that direct exposure of human cells to serum concentrations of up to 20% is still well tolerated. The complete lack of toxicity, comparable to the PBS control, is extremely rare and indicates that despite the

reactivity of this mixture, it is well tolerated by human tissues and is native to or compatible with the extracellular environments.

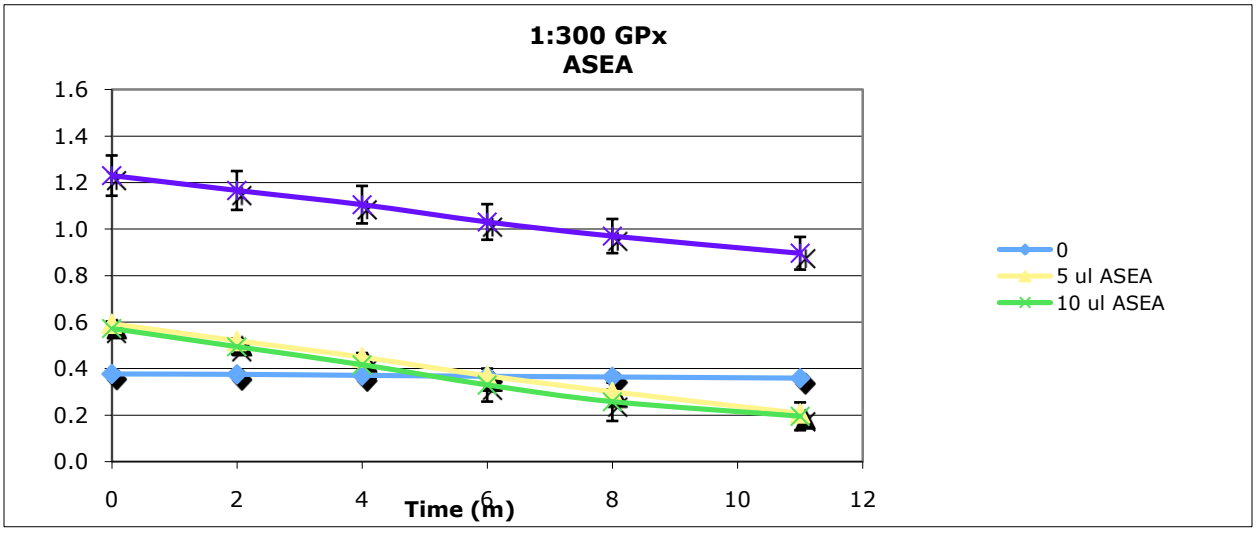
Objective 2: Determine Antioxidant Efficacy

Experimental Methods Used to Determine Antioxidant Efficacy of Glutathione Peroxidase (GPx)

Cell cultures of standard murine epidermal cells (JB6), obtained locally, were exposed to various small concentrations of ASEA (less than 1%) and PBS solution for 24 hours. Cell lysates were prepared for measurements of GPx enzymatic activity using a commercially available ELISA kit (GPx activity kit, Cat #900-158) according to directions of the manufacturer (Assay Designs, Ann Arbor, MI). Decrease of oxidants due to GPx enzymatic activity was monitored over an 11 minute period of time after a chemical agent (cumene hydroperoxide) initiated the reaction. The decrease of oxidants is an indication of antioxidant efficacy. To determine GPx efficacy at various concentrations of PBS or ASEA, three replications of oxidant residual in the samples were read every 2 min to generate the slope, indicating the decrease in relative fluorescence units (RFU)(oxidant residual) per minute.

Results and Observations for GPx Antioxidant Efficacy Test

After activation, the reduction of oxidants over time was closely linear, as seen in the graphs below (RFU units on vertical scale). A well-defined slope was established over the 11 minute interval.



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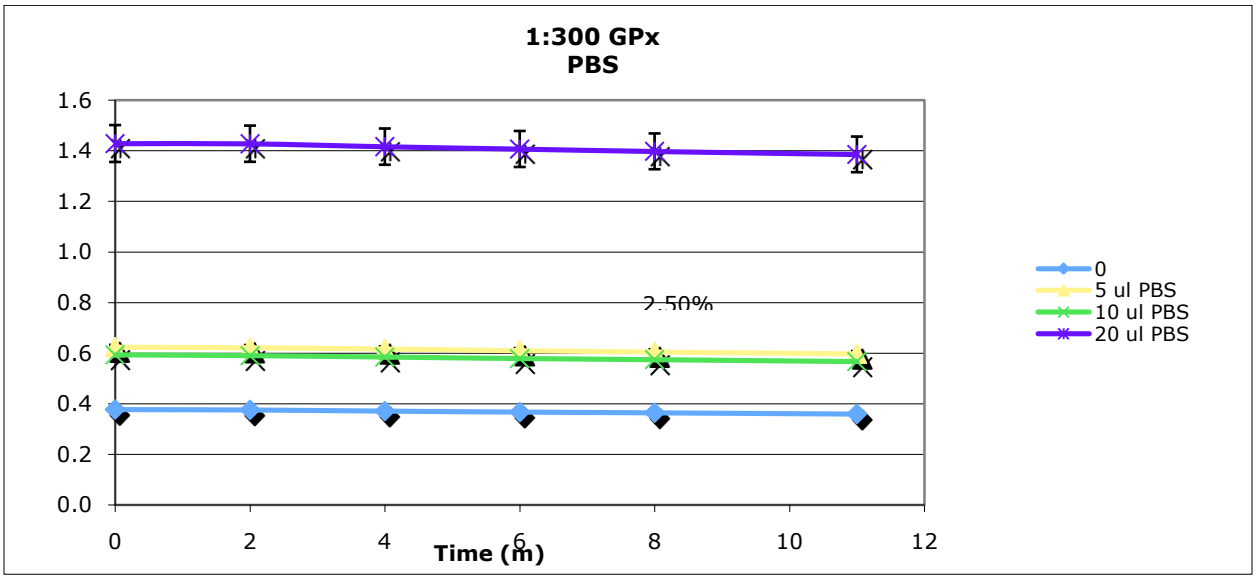
Antioxidant activity is measured by reduction of oxidants over time.



A significant increase in antioxidant activity was seen in samples infused with ASEA compared to the PBS control (second graph).

Concentration dependency, however, was not seen between the 5ul, 10ul and 20ul infusions. This suggests that GPx antioxidant activity might saturate at concentrations lower than that represented by the 5ul infusion. Such considerations will be discussed later.

The table below summarizes the data shown on the preceding graphs.

Sample Infusion Volume (< 1% total volume)	Slope for PBS Control (% reduction/minute)	Slope for ASEA (% reduction/minute)
0 ul	0.1%	0.1%
5 ul	0.1%	3.6%
10 ul	0.2%	3.6%
20 ul	0.3%	3.7%

Table comparing oxidant reduction due to ASEA infusion

The raw data reflects more than a 10 fold increase in antioxidant activity related to ASEA infusion. Taking into account experimental uncertainties, it is 98% certain that the serum infusion of small concentrations (< 1%) of ASEA increased antioxidant efficiencies by at least 800%. Further investigations should be done to confirm this increase and explore concentration dependence for these low-level serum concentrations.

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Experimental Methods Used to Determine Antioxidant Efficacy of Superoxide Dismutase (SOD):

Human HMVEC-L cells were treated with 10% phosphate buffered saline (PBS; vehicle control), 5% or 10% ASEA for 24 hr at which time cell lysates were prepared for measurements of SOD activity using a commercially available kit (SOD activity, cat# 900-157) according to manufacturer's (Assay Designs, Ann Arbor, MI) directions. Cell culture medium was assayed for SOD activity in parallel. Limited trials with smaller concentrations of ASEA < 1% and murine epidermal cells were also attempted.

Results of First-Attempt Methods to Determine SOD activity for high serum ASEA concentration:

Diluted lysates showed a marginal increase in enzymatic activity associated with ASEA treatment. Changes in enzymatic activity were marginal in the initial range of 5-10% ASEA (final concentration, v/v). The data represent the first attempt to measure SOD activity using primary HMVEC-L cells treated with ASEA. It is feasible that the lack of SOD activity associated with 5-10% ASEA might be related to non-specific inhibition at high dose. The primary concern is that we have little understanding of the primary human HMVEC-L cell model and cannot determine whether these cells are optimal for investigating antioxidant defense regulation induced by ASEA. For example, ascorbic acid, known to break down certain redox signaling complexes in ASEA, is supplemented into the medium and it is feasible that some modification of the medium formula (such as omission of ascorbic acid for short periods of time defined empirically) could produce more optimal conditions for detecting antioxidant defense regulated by ASEA. Initial efforts to serum-starve these cells, as one approach to increase sensitivity and optimize the model, were unsuccessful and resulted in extensive cell death over 24 hours, indicating that the cells are dependent on the growth factors supplemented in the cell culture medium to maintain cell viability. If we interpret the initial ASEA concentrations (5-10%) to be high (based on inhibition of medium enzymatic activity and cell proliferation), then it is possible that the marginal increase in enzymatic activity associated with cell lysates observed here may not accurately reflect antioxidant defense regulation possibly occurring at lower concentrations. The use of an *in vitro* model system with a well defined and robust NRF2-regulated antioxidant defense response would help address some of these uncertainties. In retrospect, we have observed that a lower

concentration of ASEA (1%) induces the nuclear translocation of the NRF2 transcription factor. In addition, the 24 hr time point was chosen for the initial screen as a general time point for *in vitro* investigations that would capture transcriptional regulation, however, this time point was not optimal.

Results of Further Investigations into SOD enzymatic activity at low ASEA concentrations (< 1%):

It was found in another investigation that NRF2 nuclear translocation (data and results are in the following sections), took place at low doses of ASEA (less than 1%) and elicited peak SOD antioxidant activity at about 30 to 120 minutes after exposure. Thus when SOD antioxidant activity was measured due to low-concentration ASEA exposure at a 30 to 120 minute time points, results similar to the GPx enzymatic activity were seen both with murine epidermal (JB6) cells and serum-starved HMVEC-L cells at a time point 90 to 120 minutes. Graphs were not supplied, however, a 500% increase in peak SOD enzymatic activity was estimated over a short 120 minute term, with 95% confidence. Specific work on low-level concentration dependence still has yet to be done.

Objective 3: To Determine Antioxidant Transcriptional Activity

Experimental Methods Used to Determine Nuclear Translocation of NRF2 in HMVEC-L Cells and Western Blot Verification:

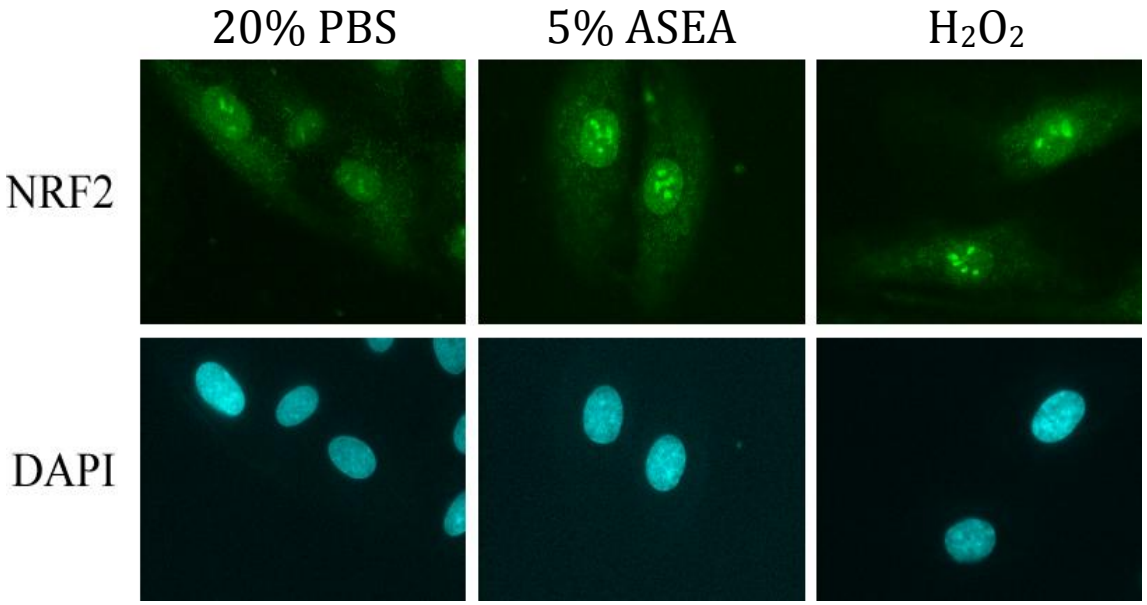
HMVEC-L cells were again thawed and maintained according to manufacturer's directions. The culture medium contained epidermal growth factor, hydrocortisone, GA-1000, fetal bovine serum, vasoactive endothelial growth factor, basic fibroblast growth factor, insulin growth factor-1 and ascorbic acid in randomly cycling cultures. Ascorbic acid was withheld from serum-starved cultures.

HMVEC-L Cell cultures in both normal random cell cycles and in serum starvation were exposed to high-concentration (5-20%) and low-concentration (1%) ASEA in the serum medium and analyzed in conjunction with cultures exposed only to phosphate buffered saline solution (PBS), as a negative control. At time points of 30, 60, 90, and 120 minutes, aliquots of cells from each of the cultures were placed under a fluorescent microscope, stained by a fluorescent dye designed to tag the NRF2 transcription factor along with the DAPI

fluorescent nuclear stain that aids the computer software to find the nuclei. Computer automated imaging techniques were used to determine the relative degree of nuclear accumulation of NRF2 via fluorescent analysis over several cells. NRF2 regulates the transcription of a number of phase II antioxidant defense enzymes and raises the possibility that additional antioxidant defense enzymes, such as glutathione transferase, may be expressed through exposure to ASEA. Thus the accumulation of NRF2 into the nucleus, as seen visually in the microscope images, is an indicator of increased antioxidant expression in the cells.

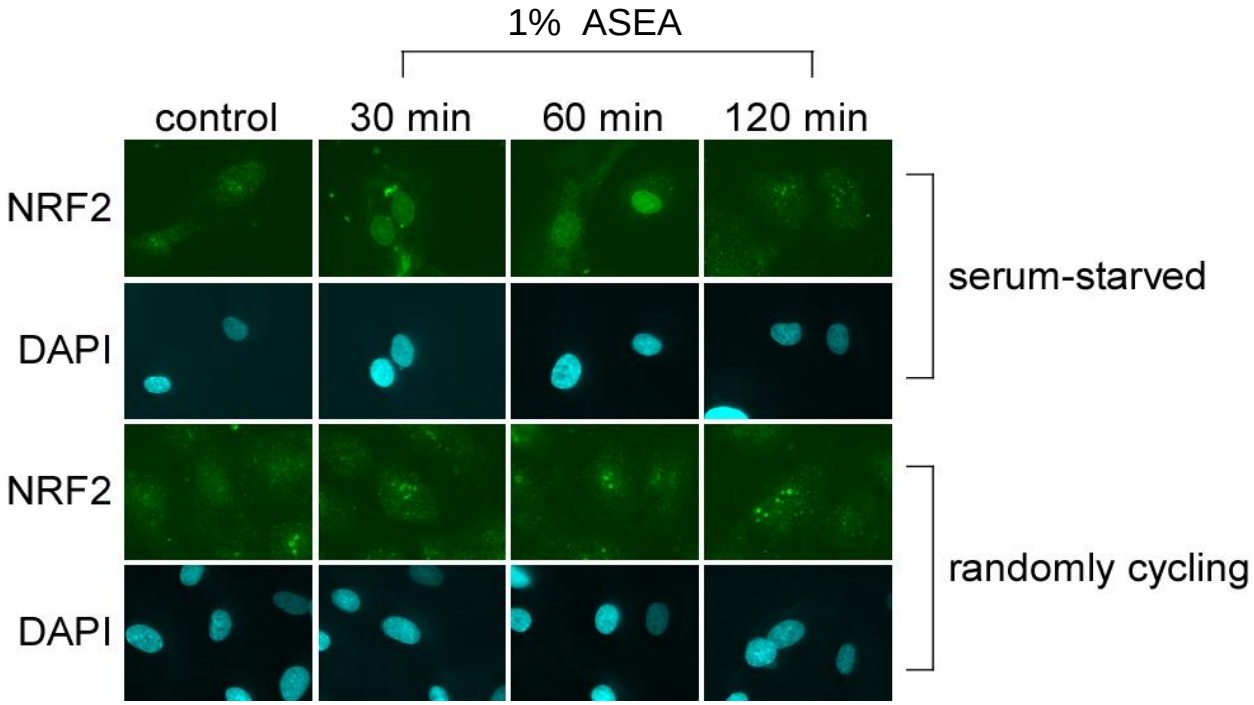
Results of HMVEC-L Nuclear Accumulation of NRF2:

Initial screen of human endothelial cells suggests a subpopulation of cells showed increased nuclear staining pattern (focal) following treatment with high-concentration ASEA. The Position of nuclei are indicated by DAPI stain in lower panel. Foci appear brighter in ASEA stimulated cells which indicates higher level of NRF2 transcription factor in the nucleus. H_2O_2 was used as positive control. This effect was difficult to quantify based on nuclear staining pattern. Validation is required by Western blot.



Typical cell images are shown below for indicated cell cultures exposed to low-concentration ASEA. Accumulation of NRF2 into the nucleus was clearly seen in serum-starved cell cultures exposed to low-concentration ASEA. Automated analysis revealed strong time-

dependent nuclear accumulation of NRF2 in serum-starved cells, relative to the negative control, at the 30 and 60 minute time points.



Nuclear NRF2 staining profiles in randomly cycling and serum-starved HMVEC-L cells.

The nuclear staining profile was qualitatively different from the cells maintained in optimal growth medium (randomly cycling group). There was weak qualitative nuclear accumulation of NRF2 induced by ASEA exposure in these cells at 30, 60 and 120 minute time points, and yet the effect was not nearly as pronounced as in the serum-starved cultures. However, serum-starvation induced significant cell death complicating interpretation of the data. The trends appeared weak and require validation by Western Blot.

Experimental Methods for Western Blot Validation of NRF2 Nuclear Accumulation:

HMVEC-L were treated with 1% ASEA, nuclear extracts were separated through centrifugal differentiation from the extra-nuclear cytosol at 30, 60 and 120 min and subjected to Western Blot analysis for NRF2. In the Western blot experiment the extra-nuclear fraction was probed for phosphorylated proteins using a combination of anti-phospho serine, threonine and tyrosine antibodies. Virtually all cellular

processes are regulated by posttranslational modifications and protein phosphorylation is a prevalent mechanism. Observable changes in protein phosphorylation can lead to a mechanistic understanding of the cellular processes perturbed by ASEA and provide a defined endpoint to better define dose-dependent regulation of cell function by ASEA in vitro, as well as provide a potential candidate molecular marker that may be used to provide in vitro-in vivo correlates. Hydrogen peroxide (H_2O_2) was included as a positive control for oxidant damage.

Results for Western Blot Validation of NRF2 Nuclear Accumulation:

NRF2 levels were increased in a time-dependent fashion in nuclear extracts prepared from HMVEC-L cells treated with 1% ASEA. H_2O_2 (30 min) did not increase nuclear NRF2 levels. In contrast, when protein phosphorylation was examined in the extra-nuclear fraction (separated from nuclei by differential centrifugation) we observed a single band by Western blot analysis and this is likely due to the dilution of the extra-nuclear fraction during the cell fractionation process (other phosphorylated proteins are obviously present but are below detection limits under these conditions) or specificity of the anti-phospho-antibodies used was insufficient to detect a broad range of phosphorylated proteins. However, we did observe a marked increase in the phosphorylation of the protein detected following H_2O_2 treatment, indicating that this phosphorylation event is highly sensitive to redox regulation or activation of protein kinase/deactivation of protein phosphatase activities subsequent to oxidative damage. Treatment of cells with 1% ASEA decreased phosphorylation levels associated with this protein in a time-dependent fashion.

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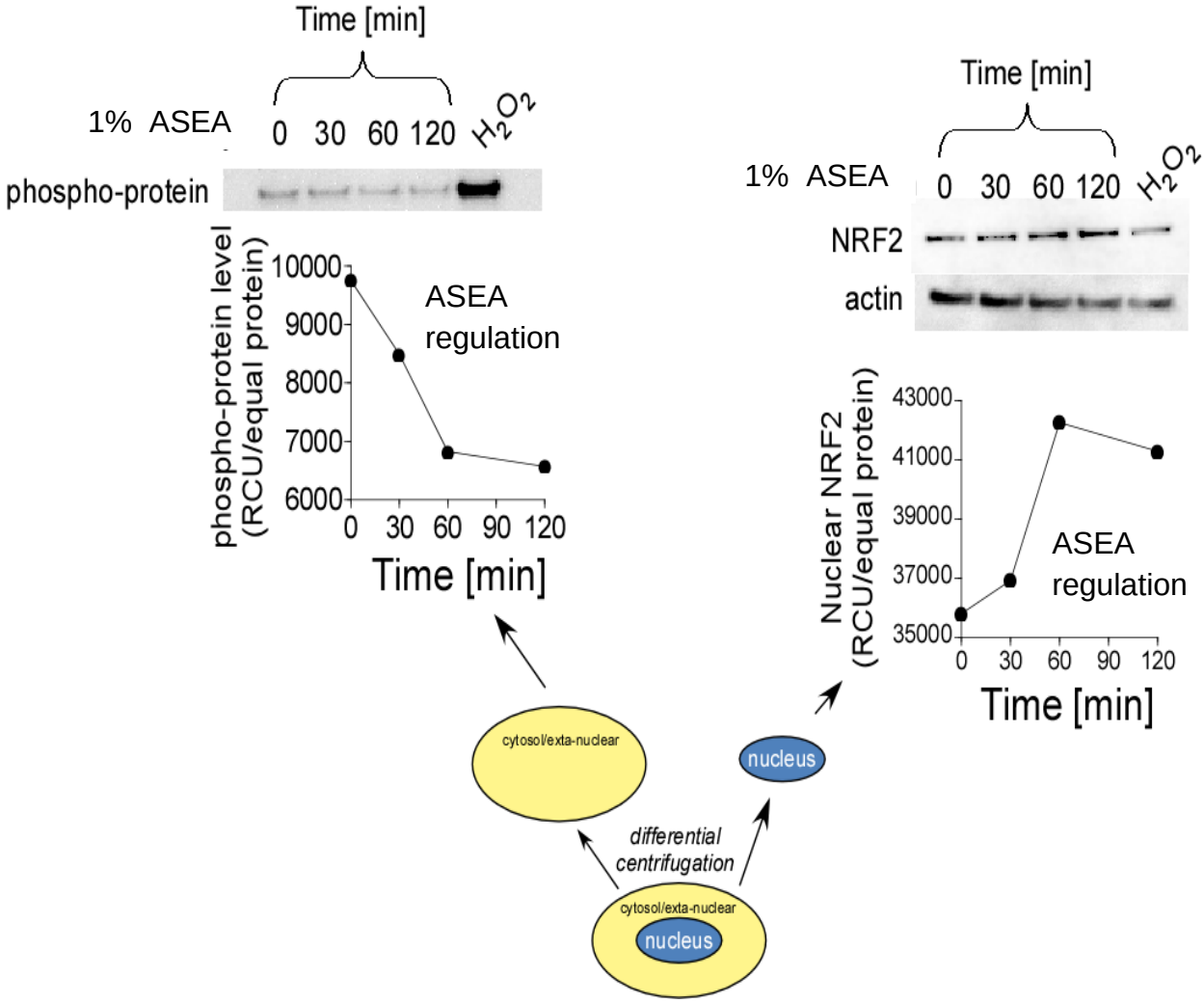


Reductions in phosopho-protein regulation in extra-nuclear fractions were seen along with strong time-dependent NRF2 accumulations in the nuclear fractions, indicating clear time-dependent up-regulation of antioxidant expression.

At this point it is worth mentioning that NRF2 activity has been clearly detected in conjunction with low-concentration ASEA exposure without the normal prior NF-kB activity. This suggests that phase II antioxidant defense mechanisms have been stimulated without the normal prior phase I toxic response. This behavior has no precedent or is extremely rare. It appears from the data that ASEA is able to stimulate antioxidant expression without ever eliciting a prior low-level phase I toxic response.

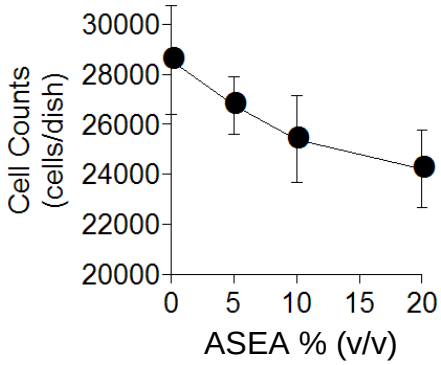
Objective 4: To Determine Cell Proliferation and Viability

Experimental Methods to Determine Proliferation of Murine (JB6) Cells and HMVEC-L Cells and LDH Activity with Exposure to ASEA:

HMVEC-L cells were treated with 5-20% ASEA for 72 hr and cell number was determined using a Coulter Counter. Control (0 concentration group) was treated with 20% PBS. Serum LDH levels were also measured as an indicator of cell culture viability at 0 to 20% ASEA serum concentrations. Recall that lower serum LDH concentrations indicate less cell membrane failure. Similar experiments were performed for murine (JB6) epidermal cells.

Results for Proliferation of Murine and HMVEC-L cells and LDH activity:

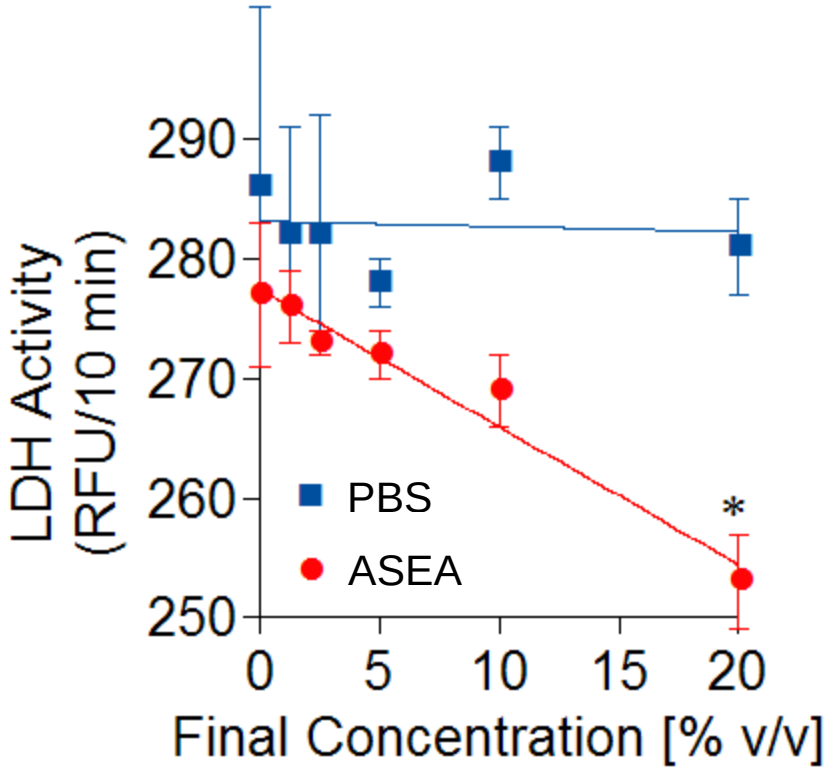
The initial in vitro screen indicates that high-concentrations of serum ASEA may inhibit cell proliferation (for both murine epidermal cells [JB6] and primary human lung microvascular endothelial cells [HMVEC-L]) in the concentration range of 5-20%. In this concentration range we also observed direct inhibition of LDH enzymatic activity. The data are somewhat contradictory as the decreasing cell counts indicate cell death, yet lower serum LDH levels indicate higher cellular membrane integrity. At the highest concentration tested (20% v/v), cell proliferation was inhibited by approximately 20%.



HMVEC-L counts indicate reduced proliferation for high-concentration ASEA after 72 hours

The mechanism behind reduced proliferation cannot be deduced and could be related to interference with growth factor responsiveness or other possible interpretations such as enhanced programmed death (apoptotic response) for damaged cells. It is noteworthy that high-concentration serum ASEA for in vitro enzymatic enhancement studies is not optimal,

it is possible that the initial screens underestimated or even missed antioxidant defense (SOD) regulation by ASEA and thus indicate that low-concentration (< 1%) ASEA and/or short exposure times should be employed for such purpose.



High-concentration ASEA exposure reduced serum LDH activity in HMVEC-L cells

Further studies were done that investigated the action of stressed cells upon exposure to ASEA; the source of stress resulting from a variety of chemical and environmental stressors. These investigations offer clues for the possible mechanisms.

Objective 5: Determine Action of ASEA on Stressed Cells

Experimental Methods to Determine cell viability of HMVEC-L exposed to various mixtures of Cachexin stressor and high-concentration ASEA:

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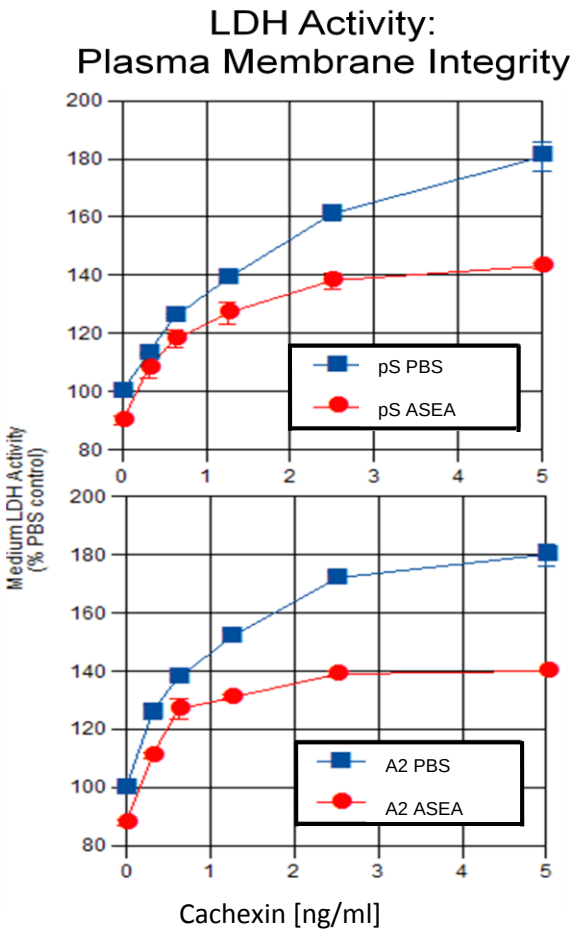
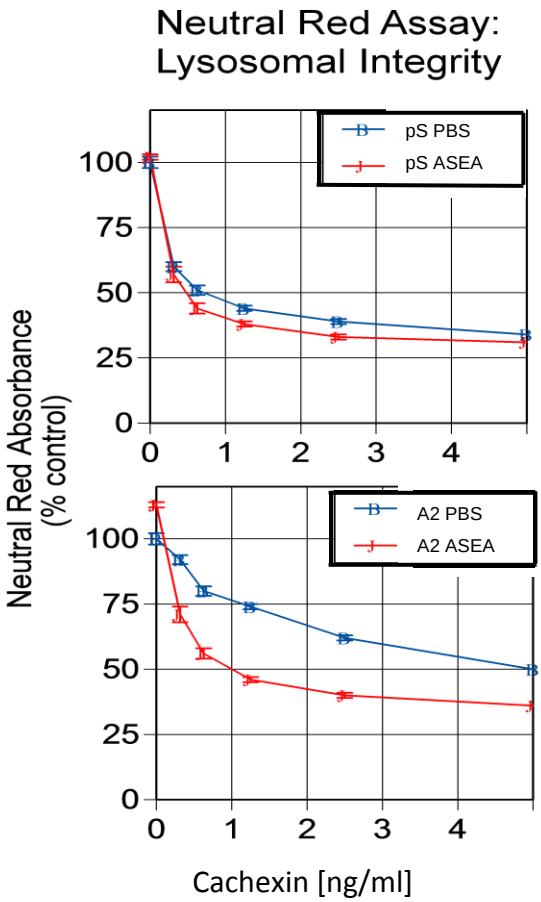
HMVEC-L cultures with normal random cell cycles (pS) and cultures approaching confluence (A2), which are generally less sensitive to Cachexin, were infused with escalating concentrations of Cachexin stressor (0-5 ng/ml). These cultures had been pretreated with either a 10% PBS control or 5-10% concentration of ASEA for 24 hours. Two indicators for cell viability were employed. Serum LDH levels were obtained as an indication of membrane integrity and Neutral Red dye was used as an indication of lysosomal integrity. Recall that as cell membranes fail, LDH is released into the serum medium. Lower quantities of LDH indicate higher cell viability. The integrity of lysosomes, necessary for viable cell function, are measured by absorption of Neutral Red dye stain. Higher quantities of Neutral Red absorbance indicate higher cell viability.

Results of HMVEC-L viability exposed high-concentration ASEA and to escalating amounts of Cachexin stressor:





Both confluent (A2) and normal (pS) HMVEC-L cultures exhibited up to 30% improvement (relative to PBS controls) in LDH levels related to ASEA exposure after acute (up to 5 nm/ml) Cachexin insult. The LDH data suggest that HMVEC-L cells stressed by Cachexin are less likely to die due to cell membrane failure after being exposed to ASEA.

Behavior of lysosomal integrity in HMVEC-L cells as measured by Neutral Red absorption exhibited behavior dependent on cell culture phase. As expected, the confluent (A2) cells in the PBS control were much less sensitive to Cachexin insult than cells in the PBS control normal random phase (pS) culture; this is evidenced in the 5 ng/ml Cachexin data: Lysosomal levels in A2 cells dropped only 50% compared to 70% in the pS culture. Exposure of the normal (pS) cultures to ASEA made little difference in lysosomal integrity under similar Cachexin insult, yet exposure of confluent (A2) cell cultures to ASEA made them much more sensitive to Cachexin insult, regressing to behavior similar to that exhibited by the normal more sensitive (pS) cells.

This is the first evidence presented that suggests that exposure of abnormal (Cachexin-insensitive) HMVEC-L cells to ASEA can make them more sensitive. The data suggest that confluent (A2) cells stressed by Cachexin are more likely to die when exposed to ASEA, these abnormal cells when exposed to ASEA exhibit closer to normal behavior in the presence of Cachexin. This behavior was initially unexpected as the hypothesis of the experiment was that ASEA would help cells protect themselves against toxic insult. As it turns out, it appears that ASEA exposure only helps normal healthy cells to protect themselves against oxidative insult and yet seems not to help cells protect themselves against Cachexin. ASEA exposure may even help facilitate the death of the stressed cells that are close to the end of their normal life cycle. Incidentally, the normal role of Cachexin in the tissues is to facilitate the death and replacement of damaged cells.

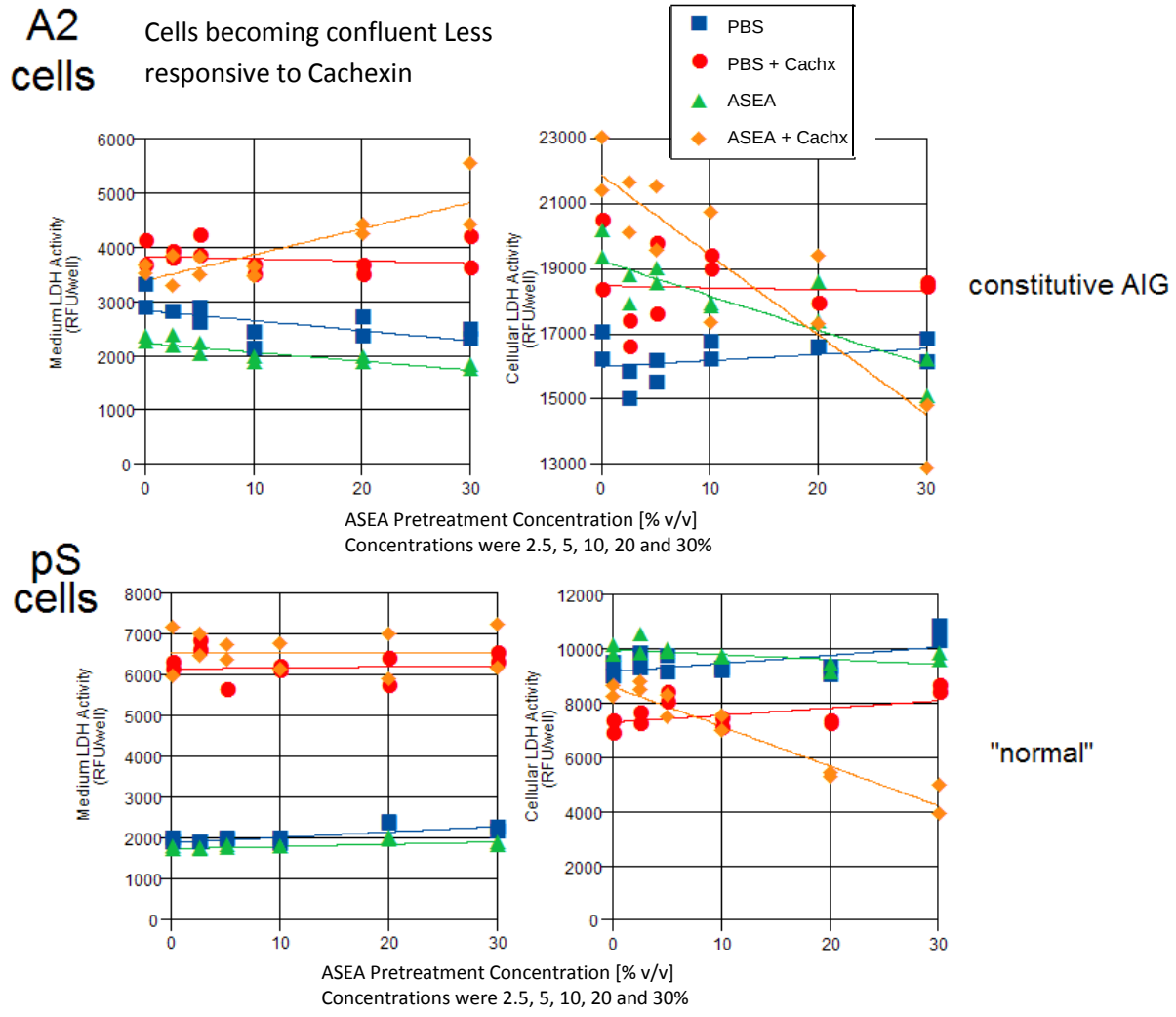
Experimental methods to determine the ASEA concentration-dependent response of A2 and pS phase HMVEC-L cells to Cachexin insult:

HMVEC-L cell cultures, prepared in two phases, in the confluent end-of-life-cycle A2 phase (a phase typically insensitive to Cachexin insult) and in the normal random cycle pS phase were exposed for 24 hours to serum concentrations (v/v of 2.5%, 5%, 10%, 15% and 20%) of either the PBS control or ASEA. Cachexin responsiveness was then determined by monitoring LDH activity in both the intracellular

cytosol and in the surrounding growth media. Recall that increased LDH activity in the growth media indicates cell membrane rupture and death (LDH release) and the decrease of intracellular LDH activity indicates loss of cellular integrity. Thus the cell cultures that are responsive to Cachexin insult would experience an increase in medium LDH activity and a decrease in intracellular LDH activity.

LDH activity in untouched cell culture controls were compared to that of cell cultures insulted with 5 ng/ml Cachexin for each ASEA concentration considered. The ASEA concentration dependence was then graphed against LDH activity for each insulted culture and control.

Results of concentration-dependent response of HMVEC-L cells to Cachexin insult:



The graphs on the left represent growth medium LDH activity and on the right represent intracellular LDH activity.

Relative to the PBS control, the Cachexin response for the normal pS cells was much smaller than expected. Only slight decreases in cell membrane integrity were seen in the PBS control cultures and the intracellular LDH activity remained the same. With ASEA exposure, by itself, the normal pS cell cultures suffered a slight decrease in overall cellular integrity and increase in cell death. It should be noted that since the large expected response of the control pS cells to Cachexin was not manifest, it is probable that the pS cell cultures used in this investigation were nearing a confluent or non-responsive state.

There was, however, a clear response when Cachexin insult was added to the pS cell cultures exposed to various ASEA concentrations, cultures demonstrated a clear loss of intracellular LDH function and integrity. However, the accompanying indication of cell death was not seen. This seems to indicate that the "normal pS" cells were made more sensitive to Cachexin reception by ASEA exposure, yet not brought completely to the point of cell death.

The A2 cell culture response was very clear. ASEA exposure, even without Cachexin, seemed to cause loss of intracellular LDH integrity, though it did not affect cell death. However, when Cachexin insult was applied to such A2 cultures, ASEA exposure clearly amplified the Cachexin reception rapidly decreasing cellular function and there were also clear indications of concentration-dependent cell death. There is strong evidence that ASEA exposure increases Cachexin responsiveness in the A2 cell cultures.

The results imply that ASEA exposure significantly increases Cachexin responsiveness in A2 and borderline pS HMVEC-L cell cultures. Of possible interest, ASEA exposure alone might decrease integrity of cellular LDH activity in A2 type cells; recall that zero toxic response was detected in randomly cycling cells even under large concentrations, so effects due to toxicity are not expected in normal cells. It appears that ASEA exposure may tend to accelerate the removal of non-responsive confluent cells. This is evidently true when Cachexin is present. These results might also bear on the observations that ASEA exposure seemed to diminish cell proliferation in high concentrations. No such trend was tried for low-concentration exposure. Note that it is difficult to discount the possibility that

high-concentration effects might simply be artifacts due to the interference of ASEA with the growth medium.

Experimental methods to determine effects of 5-10% ASEA exposure to cells stressed by radiation and serum starvation:

Murine (JB6) cell cultures were subjected to high-level radiation exposure (X-rays) and, in a separate investigation, cultures were subject to serum starvation of growth factors for 24 hours. The cells were then exposed to 5-10% ASEA exposure as means to determine the effect of ASEA exposure on such stressed cells. Cell counts were taken before and after ASEA exposure.

Results of effects of 5-10% ASEA exposure on radiation and serum-starved murine cells:

Quantitative analysis was not compiled for these experiments. Qualitative analysis, however, reveals results that might be of some interest. For the radiation-damaged culture, immediate cell death was observed for more than half of the culture upon exposure to ASEA. No further cell-death was seen thereafter. Upon inspection under a microscope, the remaining living cells appeared normal and healthy. It appears that ASEA exposure may have helped accelerate cell death among the more seriously damaged cells and allowed for the survival of healthy or repairable cells.

For serum-starved cell cultures similar observations were made, except the cell death was not nearly as severe, amounting to less than roughly a 20% loss. Surviving cells appeared to be very robust and viable. Similar losses, however, were also seen in serum-starved cultures that were not exposed to ASEA in later experiments. Quantitative analysis was not done being that the sudden cell death was not expected or understood at the time of the experiment. It is also a possibility that there might be some confounding factors that were not considered in these quick investigations, such as interference with growth media. Results, however, were included for completeness.

White Paper on In-Vitro Bioactivity of ASEA™ Related to Toxicity, Glutathione Peroxidase, Superoxide Dismutase Efficacy and Related Transcription Factors

June 22, 2010

General Observations and Conclusions

Brief Summary

A better understanding of the bioactivity of a certain mixture of redox signaling molecules has been determined from in vitro studies involving direct contact of ASEA with viable living HMVEC-L human cells and murine epidermal JB6 cells. Five specific objectives were pursued to determine:

1) In vitro toxicity (based on NF-kB, P-Jun translocation)
2) Effects on antioxidant efficacy (for GPx and SOD)
3) Effects on antioxidant transcriptional activity (NRF2)
4) Effects on cell proliferation and viability (cell counts)
5) Effects on stressed cells (Cachexin, radiation, starvation)

Brief Summary of Results for Objective (1):

No toxic response was observed for any healthy cell culture in normal random phases(HMVEC-L or JB6) upon exposure to high concentrations (up to 20%) of serum ASEA. Two methods were used to determine toxic response, the translocation and accumulation of NF-kB and P-Jun in the nuclei. Both of these methods are known to be sensitive to low-levels of toxicity, as verified by the positive control. A complete lack of toxic indication and/or inflammatory cytokines was observed.

Brief Summary of Results for Objective (2):

An 800% increase in GPx antioxidant efficacy in HMVEC-L cells was seen after 24 hours exposure from low-concentration ASEA (no concentration dependence seen). A transitory increase of up to 500% was seen in SOD antioxidant efficacy between 30 to 90 min. again after exposure to low-concentration ASEA (< 1%). In both cases, the low concentrations of ASEA were comparable to blood concentrations possible from oral dosing, though data is not available to confirm this. Concentration dependence at very low concentrations might be seen if such was carefully investigated.

Exposure to high-concentration ASEA, in comparison, elicited only a small relative increase in GPx antioxidant efficacy that was not concentration dependent. An increase in SOD efficacy was not seen for either high-concentration ASEA or after long (24 hr) exposures. In subsequent investigations, this information will be used to determine optimal concentrations and time points to study concentration dependence (less than 0.1% and 0-120 minutes).

Brief Summary of Results for Objective (3):

Studies examining the nuclear translocation of redox responsive transcription factors suggest that ASEA at a lower concentration (less than 1%) induces a 20-30% increase in the nuclear translocation of the NRF2 transcription factor in HMVEC-L cells that appears to be transient (30-60 min). We also observed that ASEA induced a parallel decrease in the phosphorylation of an extra-nuclear protein whose phosphorylation status is clearly increased in response to hydrogen peroxide treatment, consistent with an antioxidant mode of action.

Serum-starving HMVEC-L cells, as an approach to increase sensitivity, significantly increased the nuclear NRF2 signal induced by ASEA (1%). However, serum-starvation induced significant cell death complicating interpretation of the data.

Brief Summary of Results for Objective (4):

Cellular proliferation for both HMVEC-L and JB6 cell types (determined from cell counts) was inhibited by high concentrations (5-20% v/v) of ASEA exposure. The HMVEC-L inhibition was clearly concentration dependent, with a 20% loss of cell count at 20% ASEA concentration. In contrast to decreased proliferation, serum LDH levels significantly decreased with ASEA concentration between 5-20%, indicating increased cell membrane integrity. The results seem to indicate that cellular proliferation is decreased while cell membrane viability is increased at high concentrations. The mechanism behind such behavior cannot be deduced from the data, yet further evidence will be seen in the next section.

Brief Summary of Results for Objective (5):

The response of HMVEC-L cells when stressed with Cachexin depends upon cell phase. Normal randomly cycling HMVEC-L cells (pS) exhibited typical behavior when stressed with Cachexin: exhibiting decrease in cell viability accompanied by cell death. Confluent end-of-life-cycle (A2) and borderline HMVEC-L cells, as expected, were less sensitive to Cachexin insult, exhibiting less pronounced decreases in cell viability and less cell death.

Exposure to ASEA caused no significant change in the response of the normal random cycling pS cells to Cachexin (showing similar loss of cell viability and cell-death). However, A2 cell cultures exposed to ASEA exhibited increased sensitivity to Cachexin, restoring behavior similar to that of normal cells. This behavior was reinforced as ASEA

concentration dependence was examined. Borderline A2 cells, exhibiting a relatively small Cachexin response, and A2 cells that are normally insensitive to Cachexin insult, exhibited a much stronger response to Cachexin when exposed to ASEA, both in decrease in viability and increased cell death.

It appears that exposure to ASEA causes increased rates of A2 cell death, enhancing the natural reception of Cachexin in such end-of-life-cycle cells. Yet exposure to ASEA is not expected to cause any change in normal cell viability.

Cachexin is normally secreted to instigate cell death in damaged or dysfunctional tissues, allowing surrounding healthy cells to divide and fill in voids. Thus, increasing the sensitivity to Cachexin in dysfunctional cells may help accelerate such a process and is not always deleterious.

Acceleration of cell death was also seen in tissues that were stressed with radiation and serum-starvation associated with exposure to ASEA.

Interpretations and Conclusions

The infusion of a certain balanced mixture of redox signaling molecules, ASEA, into viable HMVEC-L and JB6 cell cultures has been seen to elicit distinct bioactivity. No indications of toxicity or the expression of inflammatory cytokines were observed and yet there was increased antioxidant and protective enzyme expression (as evidenced by increased nuclear NRF2) and greatly increased efficacy for the two master antioxidants, GPx and SOD. This behavior suggests that ASEA infusion might tend to induce and enhance oxidative defense mechanisms without inducing toxic or inflammatory responses in such cells. Such action is unprecedented or extremely rare. Normally, low-level toxicity induces slight oxidative stress and inflammatory response which in turn induces oxidative defense and cell repair mechanisms. It would be of interest to determine concentration dependency of this effect with ultra-low-concentration ASEA infusions.

The induction of cell death in cultures of dysfunctional, stressed or damaged cells by ASEA infusion should also be explored. Natural healing processes involve a repair or replace mechanism by which marginally damaged cells are repaired, when possible, or undergo apoptosis, programmed death, if they cannot be repaired and then are replaced through mitosis of healthy neighboring cells. It is fairly evident that ASEA infusion, of itself, is not causing direct stress to

exposed cells, however, it might tend to increase the efficiency of certain cytokine "death domain" messengers (Cachexin) that are designed to induce cell death in dysfunctional or damaged cells. The nuclear translocation of NRF2 can be considered part of the phase II oxidative defense response which includes expression of antioxidants, DNA repair molecules and other known repair mechanisms.

Apoptosis is part of the replace mechanism when cells have undergone unrepairable damage and must be removed and replaced. Both antioxidant defense and apoptotic mechanisms are central to normal tissue repair and regeneration. Redox signaling is involved in several of the pathways, such as p53 gene expression, that can determine whether a cell undergoes apoptosis or not. Chronic oxidative stress tends to favor cell death. Certainly the presence of Cachexin and other death domain messengers favor cell death. The observation that ASEA infusion enhances Cachexin reception might indicate that ASEA infusion also might serve to enhance reception of messengers in the signaling process that determines whether defense, repair or replace mechanisms are activated. These preliminary studies have been productive and offer many opportunities for further investigation.

Suggested Follow-On Experiments

The inhibition of cell proliferation and enzymatic activity in the 5-20% concentration range raises the possibility that this range may be too high for *in vitro* investigations. The apparent activation of NRF2 at 1% ASEA is consistent with this interpretation and suggests that the concentration-response should be extended to lower concentrations. The HMVEC-L cells are difficult to work with and not amenable to the typical experimental modifications used to enhance sensitivity, such as serum-starvation. There also appears to be a significant basal level of NRF2 transcription factor in the nucleus of control cells. If the standard cell culture conditions needed to maintain viability of primary human HMVEC-L cells is inducing activation of NRF2 and NRF2 is playing an important role in regulating the antioxidant defense response to ASEA, then HMVEC-L cells may not be optimal for this line of investigation. Comparison of antioxidant defense regulation by ASEA in HMVEC-L cells with additional attempts to modify sensitivity (such as modifying medium supplements such as ascorbic acid), as well as a comparative study with a well established model showing robust NRF2-dependent antioxidant defense regulation would help address these variables.

The use of a global response assay, such as a microarray analysis of gene expression could provide clues in this regard, but would be better coordinated when the model has been optimized for a more robust response to ASEA. Therefore, the redox indicator dyes have the advantage of reporting general cellular redox status without need for knowing the detailed molecular basis of the redox regulation. It is suggested that studies be initiated to evaluate several fluorescent indicators of redox status to identify the most sensitive flours demonstrating a response to ASEA over a broader concentration range (1% and lower). These studies can include challenge with inflammatory stimuli (at an appropriate challenge level) to produce a more robust redox response and determine whether this improves sensitivity in the detection of antioxidant defense regulated by ASEA. In parallel, additional cell lines that are well characterized for NRF2-dependent antioxidant defense regulation can be evaluated to determine the relative importance of this transcription factor to the antioxidant defense response to ASEA. Protein phosphorylation profiles may also yield specific molecular targets that are redox regulated (H_2O_2) and whose phosphorylation is reduced by ASEA. If properly developed, phosphorylated proteins could provide a specific and quantitative assay for the antioxidant defense properties associated with ASEA and could be used to bridge *in vitro – in vivo* models.