Polyphenolics
Realizing the health benefits of the grape

Why Polyphenolics?

All Grape Seed Extracts are not created equal.

Founded in 1996, Polyphenolics is a science driven organization dedicated to researching and developing innovative products using grape seed derived polyphenols to deliver specific, documented health benefits.

As a division of Constellation Brands, one of the largest wine companies in the world, Polyphenolics has access to an abundant supply of California grown grapes, and controls the entire manufacturing process, from the initial selection of grapes to the final extraction of finished material that is MegaNatural® grape seed extracts.

Polyphenolics goes beyond federally mandated traceability requirements, documenting all aspects of growing, treating, and processing the grapes.

Polyphenolics believes in forming close partnerships with our customers and remains available to customer partners during all phases of product development for technical questions and support.

What Differentiates MegaNatural® Grape Seed Extract From Others?

- Four US Patents Issued
- World-Wide Patents Pending
- Unique Composition
- Original Research
- Clinically Shown
- Condition-Specific
- 100% California-Grown Grapes
- 100% Pure Grape Seed Extract
- 100% Water Soluble
- Hot Water Extracted
- Relentlessly Tested
- FDA No-Objection GRAS

These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.

Why you should not use or consider less expensive/inferior quality grape seed extract in your product.

Polyphenolics' MegaNatural® grape seed extracts are adulterant free.
MegaNatural® grape seed extracts contain 100% grapes. Nothing more.

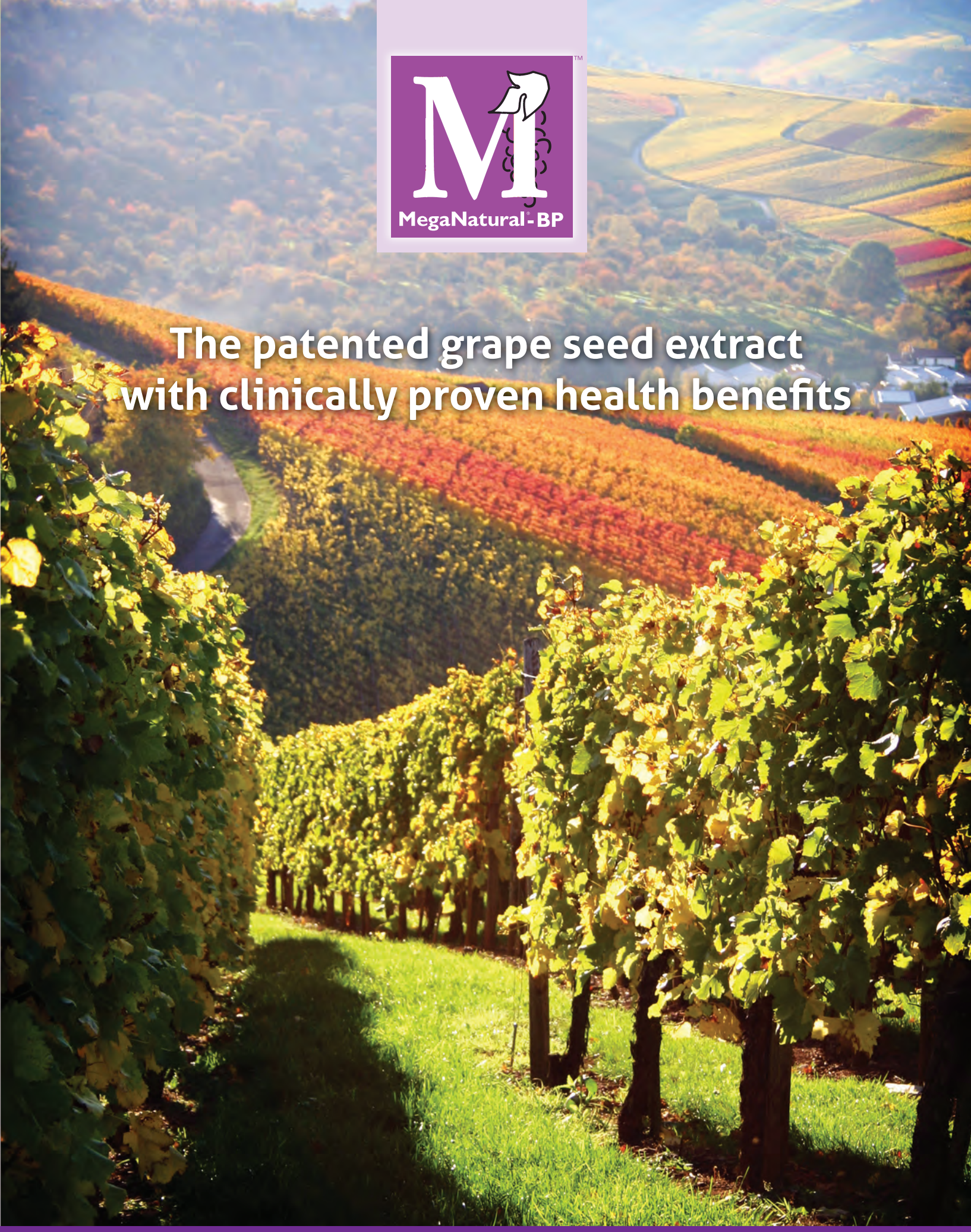
Do you know where your grape seed extract comes from?
Polyphenolics does. MegaNatural® grape seed extracts come from 100% California grown grapes traceable to the fields they were grown in.

Consistency is Extremely Important.
The consistency of every batch of MegaNatural® grape seed extracts are verified through specialized HPLC testing. Consistent activity guarantees consistent results in your finished product.

Polyphenolics invests in original research.
Polyphenolics has invested heavily in original research at both the laboratory and clinical levels. Original research is important because multiple factors, from the grape varietals chosen, to the time of harvest, and the extraction technique, can influence the constituent profile of the finished product. The only way to know if a particular extract has biological efficacy is to test the specific composition of the extract.

"Comparing Polyphenolics' grape seed extract with other grape seed extract is like comparing….Well, you get the picture."

To learn more about using MegaNatural® grape seed extracts in your dietary supplements, or functional foods or beverages, call 866-308-7678 or visit us at www.polyphenolics.com.


MegaNatural®-BP
The patented grape seed extract
with clinically proven health benefits

MegaNatural®-BP:
The patented grape seed extract with clinically proven health benefits

One of the key indicators of a healthy heart is healthy blood pressure. But what is blood pressure exactly, and why is it so important?

Blood pressure measures the amount of force exerted against the walls of the arteries in response to the pumping action of the heart. The intensity of that force depends on the volume of blood being pumped and the flexibility of the arteries.

Like a balloon being filled with water, pressure rises when the arteries contain a large amount of blood. Similarly, when the arteries lose some of their natural balloon-like flexibility, they can no longer expand easily to accommodate increased blood flow — again causing an increase in pressure.

Blood pressure is measured in two numbers: the top number represents systolic blood pressure (the force when the heart contracts); the bottom number represents diastolic blood pressure (the force when the heart rests). Anything at or below 120 mmHg/80 mmHg is considered healthy.

Why Maintaining Healthy Blood Pressure Is So Important

When blood pressure is healthy, the heart can pump blood at a relaxed pace. Once pressure in the arteries rises, the heart has to work harder to keep blood flowing. This isn't a problem if the increased demands on the heart are occasional, such as during intense exercise. However, forcing the heart to pump hard all the time puts tremendous stress on such an important organ.

As mentioned previously, arterial inflexibility leads to increased blood pressure. Unfortunately, the opposite is also true: elevated blood pressure causes the arteries to become even stiffer. Thus a vicious self-perpetuating cycle is initiated.

The Promise of MegaNatural®-BP

Fortunately, there is a natural way to help maintain blood pressure levels within a healthy range: polyphenols.* Naturally occurring in fruits, vegetables and red wine, polyphenols are a class of phyto-nutrients that have been scientifically demonstrated to support cardiovascular health.* One of the largest natural depositories of polyphenols is grape seed extract.

Manufactured exclusively by Polyphenolics, a division of Constellation Brands — the world's leading premium wine company — MegaNatural®-BP is a patented grape seed extract with clinically proven benefits. In fact, two placebo-controlled human clinical trials conducted by researchers at the Department of Preventative Cardiology, University of California Davis School of Medicine, have found that MegaNatural®-BP supports blood pressure within the normal range.* No other grape seed extract can say the same, since MegaNatural®-BP has a unique structure and composition.

The MegaNatural®-BP Advantage:
MegaNatural®-BP versus Commodity Grape Seed Extracts

Grape seed extracts abound. Only MegaNatural®-BP delivers all of the following advantages:

EFFICACY

- **Clinically Researched**

  MegaNatural®-BP is backed by two placebo-controlled human clinical trials showing it supports blood pressure within the normal range.* Importantly, additional research shows that commonplace grape seed extract does not have the same effect.

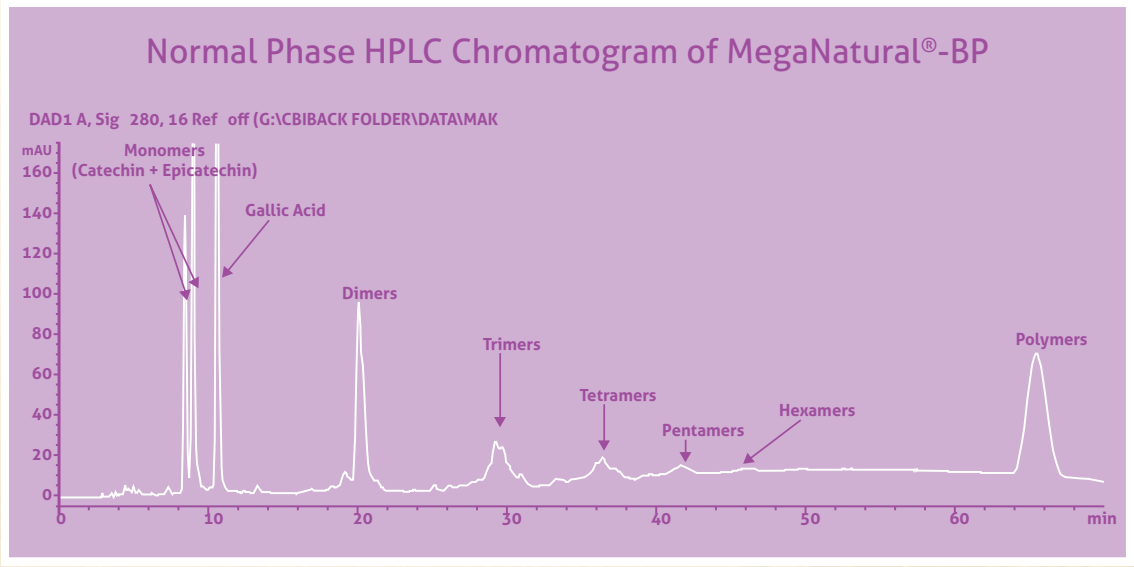
- **U.C. Davis-associated**

  MegaNatural®-BP has the exclusive privilege of being associated with the Department of Preventive Cardiology at U.C. Davis, where researchers have conducted several clinical and in vitro studies on the ingredient.

ACTIVITY

- **Structurally Unique**

  By investing heavily in research that identifies and "captures" the molecular structures within grape seed shown to provide specific health benefits, Polyphenolics has developed a grape seed extract that is structurally unique, providing 90-95% total polyphenols — the biologically active constituents.



MegaNatural®-BP is structurally unique, providing 90-95% total polyphenols (as monomers, oligomers, and polymers).

- **Improved Bioavailability**

  Because MegaNatural®-BP is selectively extracted to include a higher percentage of lower molecular weight polyphenols, it has improved bioavailability and greater absorption compared to other grape seed extracts.

* This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease.

QUALITY

- **Vertically Integrated**
  As a division of Constellation Brands, Polyphenolics has access to an abundant supply of quality U.S.-grown grapes and maintains total control over the entire manufacturing process, from raw material procurement to final extraction.
- **Environmentally Friendly**
  The relationship between Polyphenolics and its parent company results in a uniquely sustainable model of agriculture wherein all parts of the grape are utilized. The grape juice is used to make wine, the unfermented grape seeds are used to manufacture MegaNatural®-BP and the grape skin and pulp provide the raw materials for producing MegaNatural®-GSKE Grape Pomace Extract.

SAFETY

- **Non-toxic, Non-clastogenic**
  MegaNatural®-BP has been the subject of two published safety studies: a three-month oral toxicity study in rats and a high-dose clastogenic study in mice (clastogens are substances that cause damage to chromosomes). MegaNatural®-BP was shown to be non-toxic and non-clastogenic.
- **GRAS (Generally Recognized As Safe)**
  After submitting the above safety studies to the FDA, MegaNatural®-BP received No-Objection GRAS status, making it safe for use in foods, medical foods and beverages, in addition to dietary supplements.
- **Free of Side Effects**
  Clinical studies confirm MegaNatural®-BP is free of side effects, making it a safe and reliable option for people looking to maintain blood pressure within a healthy range.*
- **Relentlessly Tested**
  Every lot of MegaNatural®-BP is rigorously tested for heavy metals, pesticides and microbiological contaminants to ensure purity and safety.
- **Chemical-free**
  MegaNatural®-BP is manufactured through a patented hot-water-based extraction process, without any traces of toxic chemicals or solvents in the final ingredient.
- **Safe History**
  Millions of MegaNatural®-BP capsules have been sold in the market for the past five years, providing further evidence of its safety.

RELIABILITY

- **100% Water-soluble**
  MegaNatural®-BP is 100% water-soluble, so it can be easily added to functional beverages without dissipating out of solution.
- **Highly Consistent**
  The consistency of every batch of MegaNatural®-BP is verified through specialized HPLC testing, the accepted protocol for measuring polyphenol profiles. Consistent activity guarantees consistent results.

DIFFERENTIATION

- **Nationally Recognized**
  MegaNatural®-BP is the recipient of the prestigious Frost & Sullivan 2010 North American Product Differentiation Excellence of the Year Award, "in recognition of Polyphenolics' sharp focus on research and development, technological process innovation, and associations with major research organizations and universities resulting in a strikingly differentiated grape seed extract."
- **Intellectually Protected**
  MegaNatural®-BP is protected by a portfolio of intellectual property, including four patents — a production flow process patent (U.S. patent No. 6,544,581), a method of use patent (U.S. patent No. 7,651,707), a composition patent (U.S. patent No. 7,767,235), an exclusive production process patent (U.S. patent No. 8,075,929), — and a trademarked brand name. Several patents have also been issued worldwide. This portfolio clearly differentiates MegaNatural ®BP from commodity grape seed extracts.

Original Research on MegaNatural®-BP

While it may come as a surprise, the vast majority of raw materials in the marketplace today rely on "borrowed" science — studies performed on materials other than their own — to support their claims. MegaNatural®-BP is the rare exception.

As a science-driven organization, Polyphenolics has invested heavily in original research on MegaNatural®-BP at both the laboratory and clinical levels. Why is original research important? Because multiple factors — from the grape varietals chosen, to the time of harvest, to the extraction technique — can influence the constituent profile of the finished product. The only way to know if a particular extract has biological efficacy is to test the specific composition of the extract.

Polyphenolics is fortunate to be associated with the Department of Preventive Cardiology at U.C. Davis, where researchers have conducted numerous clinical and in vitro studies on MegaNatural®-BP.

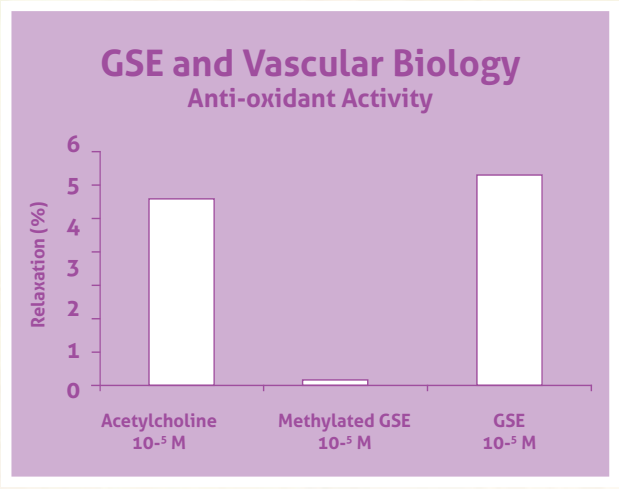
Mechanism of Action

Edirisinghe I, Burton-Freeman B, Tissa Kappagoda C. Clin Sci (Lond). 2008 Feb;114(4):331-7.

How does MegaNatural®-BP work?

Research using animal models indicates that it activates the enzyme nitric oxide synthase (eNOS) to produce nitric oxide (NO).

NO is a gaseous compound that acts as a cellular messenger. When NO is present in the inner lining of the blood vessels, known as the endothelium, it causes the surrounding smooth muscle to relax. This endothelium-dependent relaxation of the blood vessels then supports healthy blood flow.*



A study in rabbit aortic rings shows MegaNatural®-BP causes a relaxation of the blood vessels which is similar to that elicited by acetylcholine – a known vasodilator*.

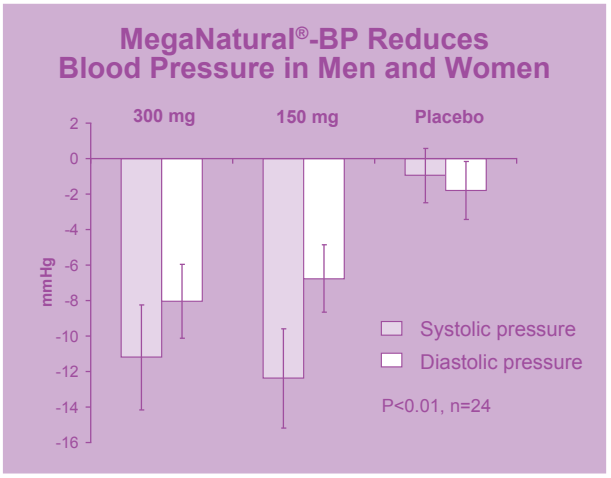
Human Clinical Trial #1

Sivaprakasapillai B, et al. Metabolism Clinical and Experimental 58 (2009):1743-1746.

Study Type: randomized, double-blind, placebo-controlled

Methodology: Twenty-five subjects were randomized into three groups: a.) placebo, b.) 150 mg MegaNatural®-BP per day and c.) 300 mg MegaNatural®-BP per day. Each group took their respective treatment for four weeks.

Results: Blood samples showed MegaNatural®-BP to be well-absorbed after a single dose, with a steep rise in polyphenols 90 minutes after ingestion. At the end of four weeks, both dosages of MegaNatural®-BP were found to help maintain blood pressure levels (both systolic and diastolic) within the normal range.* In addition, both dosages decreased levels of oxidized LDL cholesterol, with the 300 mg dosage reaching statistical significance compared to baseline. Low levels of oxidized LDL are correlated to arterial health.*



MegaNatural®-BP was found to help maintain blood pressure levels within normal range.*

Human Clinical Trial #2

Robinson M, Lu B, Kappagoda T., Journal of Pharmacy and Nutrition Sciences, 2012, 2, 155-159

Study Type: randomized, double-blind, placebo-controlled

Methodology: All 30 subjects began the trial by taking a placebo for two weeks. Then, they were randomized into two groups: a.) placebo and b.) 300 mg MegaNatural®-BP per day. Each group took their respective treatment for eight weeks.

Results: At the end of eight weeks, MegaNatural®-BP was found to help maintain blood pressure levels (both systolic and diastolic) within the normal range.*

About Polyphenolics

Founded in 1996, Polyphenolics is a science-driven organization dedicated to researching and developing innovative products using grape-seed-derived polyphenols to deliver specific and documented health benefits.

Vertical Integration

As a division of Constellation Brands, Polyphenolics has access to an abundant supply of fresh wine grapes, grown in California's Central Valley, and retains complete control over the entire manufacturing process — from the initial selection of wine grapes to the final extraction of finished material. The company goes beyond federally mandated traceability requirements, documenting all aspects of growing, treating, and handling the varietal grapes. Through painstaking supply chain documentation and laboratory testing, Polyphenolics can substantiate freshness, identity, domestic origin, and absence of chemical contaminants and genetic modification.

Dedication to Science

Polyphenolics has invested considerable resources into identifying the particular molecular structures within grape seed that provide the greatest health benefits. In addition, Polyphenolics relentlessly tests its products for:

- Efficacy, through human clinical trials
- Mechanism of action, through laboratory research
- Consistency, through HPLC testing
- Safety, through microbiological, pesticide and heavy metal testing

Social and Environmental Responsibility

The relationship between Polyphenolics and its parent company Constellation Brands results in a uniquely sustainable model of agriculture, wherein seed, juice and pomace products from wine production — which would normally be thrown away — are used to develop healthful extracts for nutritional purposes. Additionally, the company is dedicated to reducing its carbon footprint by utilizing solar power to generate much of the electricity needed to operate its facilities.

The Family of MegaNatural® Ingredients

In addition to its flagship ingredient MegaNatural®-BP, Polyphenolics offers an entire family of ingredients under the MegaNatural® brand name.

MegaNatural® Gold Grape Seed Extract

A high-quality grape seed extract with a guaranteed minimum of 90% standardized polyphenols and an Oxygen Radical Absorption Capacity (ORAC) value greater than 13,000/gram

MegaNatural® Whole Red Grape Juice Extract

An extract of whole red grape juice, providing a constituent profile similar to bilberry extract, with greater than 20% anthocyanins and 45% total polyphenols

MegaNatural®-GSKE Grape Pomace Extract

An extract made from grape skins and grape seeds that boasts a high total polyphenol content (80%) and an ORAC value greater than 11,000/gram

Polyphenolics

Realizing the health benefits of the grape

P.O. Box 99 • Madera, CA 93639 • 866.308.7678
www.Polyphenolics.com
Division of Constellation Brands, Inc.

The only grape seed extract clinically proven to support healthy hearts

Your heart loves with every beat. So love it back with **MegaNatural®-BP** from Polyphenolics.

MegaNatural®-BP is uniquely positioned as the subject of two double-blind, placebo-controlled human clinical trials conducted at UC Davis. Both studies found that **MegaNatural®-BP** supports blood pressure within the normal range* – with no adverse side effects.

Manufactured through a patented extraction process, **MegaNatural®-BP** is a group of structurally unique low-molecular weight compounds with dramatically increased absorption. Along with a healthy diet, regular exercise, and achieving a desirable weight, **MegaNatural®-BP** can give your heart the clinically proven support it deserves.*

To learn more about using **MegaNatural®-BP** in your dietary supplements, medical foods or beverages, call 866.308.7678 or visit us at www.Polyphenolics.com.

- **Proprietary water extraction process** *(U.S. Patent 6,544,581 B1)*
- **Clinically proven** *(U.S. Patent 7,651,707 B2)*
- **Unique composition** *(U.S. Patent 7,767,235 B2)*
- **Exclusive production process** *(U.S. Patent 8,075,929 B2)*
- **No chemical solvents**
- **100% water-soluble**
- **FDA No-Objection GRAS**

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MegaNatural-BP

www.Polyphenolics.com

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Polyphenolics

Realizing the health benefits of the grape

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www.polyphenolics.com

Company Description

Polyphenolics was founded 20 years ago in 1996 as a nutritional ingredient business within Constellation Brands, one of the world's largest wine companies. As a division of Constellation Brands, Polyphenolics has an abundant supply of California-grown wine grapes. The Business Unit, Operations, and R&D are located in Madera, CA to facilitate shipments to Polyphenolics' customers in dietary supplements, and functional foods and beverages.

Polyphenolics is a science-driven organization dedicated to researching and developing innovative products using grape-seed-derived polyphenols to deliver specific and documented health benefits. Polyphenolics has research grants to various well-known universities to provide clinical science and development of grape extracts.

Polyphenolics markets its products under the brand name of MegaNatural®.

Key Personnel

James A. Kennedy, Ph.D., President
Steve Kupina, M.S., Director of Quality and Technology
Gregory Arabatzis, Director of Global Sales
Lance Pray, Manager of Polyphenolics Production
Jessica Ornelas, Customer Service Advocate
Debra Cerda, Marketing Specialist

GRAS

Polyphenolics' MegaNatural® grape seed and pomace extracts are FDA No-Objection GRAS.

cGMP's

Polyphenolics maintains current quality management systems including ISO, Cal OSHA programs and third party certifications. Polyphenolics retains complete control over the entire manufacturing process. Polyphenolics goes beyond federally mandated traceability requirements, documenting all aspects of growing, treating, and handling the varietal grapes. Through painstaking supply chain documentation and laboratory testing, Polyphenolics can substantiate freshness, identity, domestic origin, and absence of chemical contaminants and genetic modification.

Technologies, Patents/Intellectual Property

In 2003, Polyphenolics developed a proprietary hot water extraction process, thereby eliminating the use of industrial solvents used by other manufacturers. U.S. Patent 6,544,581 provided improved product quality in antioxidant value and solubility standards in beverage systems.

In 2006, Polyphenolics introduced MegaNatural®-BP, a unique grape seed extract which was researched for human clinicals at the University of California, Davis, shows that a 150-300 mg/day dose reduces blood pressure from 8-12mm systolic and 5-9mm diastolic after 6 weeks. US Patent 7,651,707 B2 Method for lowering blood pressure in pre-hypertensive individuals was issued.

In 2010, US Patent 7,767,235 B2 Composition of MegaNatural®-BP was granted, followed by US Patent 8,075,929 B2 Exclusive production process in 2011.

World-wide patent applications have been filed.

Peer-reviewed research publications on mechanism and clinicals have been published and can be requested from Polyphenolics or downloaded at www.polyphenolics.com.

Major Markets

Dietary Supplements, Functional Beverages, Functional Foods

Major Products

MegaNatural®-BP Grape Seed Extract - Supports Healthy Blood Pressure…
MegaNatural®-Gold Grape Seed Extract
MegaNatural®-GSKE Grape Pomace Extract
MegaNatural®-Whole Red Grape Juice Extract
MegaNatural®- Red Wine Grape Extract
MegaNatural®- Red Wine Grape Extract with *trans*-Resveratrol

Polyphenolics is currently working on expanding the MegaNatural® line of condition-specific extracts.

Global Capabilities

Global Sales:
Gregory Arabatzis
Tel: 908-654-9342, Mobile: 908-941-6535
Email: gregory.arabatzis@cbrands.com
www.polyphenolics.com

MegaNatural-BP

Please contact one of our valued distributors:

B&D Nutritional Ingredients, Tel: 800-546-6113, www.bdnutritional.com
Beck Western Brokerage, Tel: 801-973-6333
PLT Health Solutions, Tel: 973-984-0900, www.plthealth.com

Eastern Europe:

IBCC, Rijeka, Croatia
Tel: 385 (0)51 212 213, www.ibcc.hr
Email: ibcc@ibcc.hr, Skype: ibcc,nsvast.ibcc

MegaNatural™

Polyphenolics, a division of Constellation Wines U.S.

FROST & SULLIVAN

2010 BEST PRACTICES AWARD

Polyphenolics
Realizing the health benefits of the grape

The primary challenge facing the U.S. grape seed extract market originates from the influx of low-cost, sub-standard Asian grape seed extracts. A majority of these are inconsistent in quality without any proven biological effect. The other key challenge lies in their inherently bitter taste, which affects the sensory attributes of the final product thus leaving a bad aftertaste.

In order to succeed in this market, manufacturers will have to ensure a consistent supply of high-quality ingredients with proven biological efficacy and scientific validation. Companies also need to look at developing an extract, which has no aftertaste, thus giving them a critical advantage in penetrating the food and beverages market.

Criterion 1: Unique Features

Polyphenolics' sharp focus on research and development as well as technological process innovation, through tie-ups with major research organizations and universities, has resulted in a strikingly differentiated grape seed extract, MegaNatural®-BP. MegaNatural-BP is a patented grape seed extract with a clinically proven blood pressure lowering quality unlike any other competing ingredient. The company has associated itself with the department of Cardiology at UC Davis, to study the role of grape seed extracts in lowering blood pressure in patients who have metabolic syndrome and pre-hypertension. The outcome of the study (published in the July 16th issue of Metabolism Clinical and Experimental) indicated that when MegaNatural-BP is taken in conjunction with lifestyle modification, it could effectively lower blood pressure in patients with metabolic syndrome. The UC Davis study was conducted under the leadership of Dr. T. C. Kappagoda, Professor of Cardiovascular Medicine at UC Davis Health System, and its protocol has been successfully patented. The company obtains 90 to 95% polyphenols in grape seed through its unique, patented, hot water-based extraction process as opposed to using chemicals and solvents such as Acetone. This enables selective extraction of high quality bioactive phenols. In addition, this unique process also ensures that, unlike most competing products, MegaNatural-BP has no bitter aftertaste. The company's patents for the manufacturing process and the composition of matter (grape seed extract) discourages easy duplication and it secures the company's leadership position in the market place.

Criterion 2: Quality Excellence

The consistent, high-quality of the company's grape seed extracts differentiates it from a majority of other market participants, who sell generic grape seed extracts. Polyphenolics' parent company, the Constellation Brands is the largest wine maker in the world. Owing to the integrated structure, Polyphenolics has easy access to high quality grapes and it retains complete control of the entire production cycle, from initial selection of wine grapes to the final extraction of grape seed extracts. To illustrate the high quality of the MegaNatural line of grape products (MegaNatural-BP Grape Seed Extract, MegaNatural GSKE Grape Pomace Extract, MegaNatural GSKE-40 Grape Extract, MegaNatural Gold Grape Seed Extract, and MegaNatural Rubired Grape Juice Extract) the NSF International has authorized Polyphenolics to carry the NSF Certification marks on its products. This certification also signifies the excellence of its patented production process. In addition, the MegaNatural-BP has also achieved the U.S. Food and Drug Administration's No-Objection Generally Recognized as Safe (GRAS) status, thus validating that it is safe to be added to new or existing products.

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Criterion 3: Scientific Marketing and Forward-looking Strategies

Polyphenolics has penetrated the U.S. grape seed extract market through co-branding with companies such as GNC, Inc., The Vitamin Shoppe, and host of other small to mid size companies. In addition to this, it has also tied up with various multi-level marketing companies. Many of GNC's heart-health related products sport the MegaNatural-BP logo on the back. Since the initial days of research at UC Davis, Polyphenolics had started participating in promotional activities across trade fairs, symposiums, television, radio, print media, and the Web. In addition, the company does not allow usage of lower dosage (in dietary supplements) than what has been prescribed by the UC Davis study, for its MegaNatural-BP products. This uncompromising attitude has enabled the company to position itself as a reliable producer and supplier of premium quality branded grape seed extracts.

Going forward, the company plans to focus on further promotion of its products. Although initially, dietary supplements were the primary focus area for the company where it is already a well-known brand, the company is now focused on functional foods and beverages. It is presently working with two major beverage companies in this sector and expects the commercial launch of the ensuing products by 2012. The company is aiming to leverage the GRAS status further for increased penetration into the functional food and beverage market.

On the research & development front, Polyphenolics continues to explore applications related to blood pressure. It has collaborated with the Illinois Institute of Technology and is carrying out advanced research under the leadership of Dr. Britt Burton-Freeman, the Director of Nutrition at the National Center for Food Safety and Technology at IIT. The study aims to evaluate the difference in effect on blood pressure between MegaNatural-BP used in capsule form (used as a control) and in beverage form. In addition, through this trial, the company intends to detect the dosage level, which is required to control blood pressure through MegaNatural-BP in a beverage.

With such smart marketing strategies and persistent research endeavors, Polyphenolics has been able to differentiate itself from its competitors in the U.S. grape seed extract market and is poised for further growth by successfully executing these forward-looking strategies.

Criterion 4: Identifying and Targeting Consumer Needs

Metabolic syndrome is a combination of medical disorders that spur the risk of developing cardiovascular disease and diabetes. Up to 25% of the U.S. population is estimated be affected by this medical condition with Hypertension being one of the biggest issues. Polyphenolics recognized these conditions, which is why they took a scientific, research-backed approach with the MegaNatural-BP grape seed extract. This branded ingredient offers consumers an effective, beneficial, and most importantly, a natural solution for high blood pressure. A key differentiator is that MegaNatural-BP grape seed extract has no side effects, where as traditional hypertension drugs do. Under the able guidance of Dr. T. C. Kappagoda, Polyphenolics is persistently advancing its scientific research with MegaNatural-BP in order to fight other health-related challenges, such as Type II diabetes. Polyphenolics is also exploring a new grape seed extract, MegaNatural-AZ benefit potential when it comes to brain diseases. The company, in conjunction with Dr. Giulio Maria Pasinetti, of the Research Center at Mount Sinai School of Medicine, is looking at a new product line , MegaNatural®-AZ, for controlling the progression of Alzheimer's and Dementia.

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Criterion 5: Positive Brand Perception

The brand perception for Polyphenolics' products is extremely positive as their efficacy is backed by scientific studies. The company has been able to implement a "communication around science" strategy with great effect. Websites, press releases, and medical writers have been just a few significant vehicles employed by Polyphenolics to maximize company visibility in the market place. The company has been successfully reaching out to consumers through radio, publications, video, news releases, and consumer magazines. Besides its research-backed products, Polyphenolics has also been praised for its analytical approach. Its methodology for calculating total phenols has been adopted by the National Nutritional Foods Association. In addition, the company has an honorable position at the Association of Analytical Communities, for developing a newly validated analytical testing method for grape seed extracts. These attributes have added additional credibility to Polyphenolics' products, which have resulted in increased brand loyalty. As a result, over the past 18 months, the company has witnessed approximately 50% overall growth, thus transforming itself into the market leader in the U.S. grape seed extract market.

Effect of Grape Seed Extract on Blood Pressure in Subjects with Pre-Hypertension

M. Robinson, B. Lu, I. Edirisinghe and C.T. Kappagoda*

Department of Internal Medicine, University of California Davis, Davis, California, USA

Abstract: Pre-hypertension affects approximately 31% of the adult population of the United States over the age of 18 years. It is defined in the 7th report of the Joint National Committee (JNC - 7) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure as a systolic blood pressure of 120-139 mmHg or a diastolic blood pressure of 80-89 mmHg. JNC-7 also recommended that individuals considered to be prehypertensive require health-promoting lifestyle modifications to prevent cardiovascular disease. This study was undertaken to determine whether a grape seed extract (GSE) which is a nutraceutical containing vasodilator phenolic compounds lowers blood pressure in subjects with pre-hypertension. The subjects were randomized into a placebo or an experimental group (GSE at a dose of 300 mg/day) and treated for 8 weeks. Serum lipids and blood glucose were measured at the beginning of the study and at the end. The blood pressure was recorded using an ambulatory monitoring device at the start of the treatment period and at the end. Both the systolic and diastolic blood pressures were significantly lower after treatment with GSE. Treatment with the placebo had no effect on blood pressure. There were no significant changes in serum lipids or blood glucose values. These findings suggest that GSE could be used as a nutraceutical in a lifestyle modification program for patients with pre-hypertension.

Keywords: Grape seed extract, pre-hypertension, human, polyphenolics.

INTRODUCTION

Hypertension affects approximately 60% of adults in the United States [1] and remains a major cause of morbidity and mortality. Despite the availability of numerous antihypertensive medications, control of blood pressure to optimal levels remains inadequate in most patients. In people over the age of 18 years, the prevalence of pre-hypertension alone in the U.S. is 31% [1]. It is defined by the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure in its Seventh Report (JNC 7) [2] as a systolic blood pressure between 120 and 139 mmHg or a diastolic blood pressure between 80 and 89 mmHg. Current guidelines recommend that these individuals should be managed by lifestyle modifications which include exercise, weight management, salt restriction and consumption of a diet rich in fruits and vegetables [2].

There is evidence that such a regimen which includes vegetables and fruits coupled with a low fat intake has a beneficial effect on blood pressure [3]. It has been suggested that this effect is at least in part due to the presence of phenolic compounds in the plant products [4]. These compounds have also been shown to have vasodilator effects [5-7]. Of all the phenolic compounds, those derived from grape seeds appear to have received the most attention, possibly because of their involvement with the French Paradox [8].

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Previous studies completed in our laboratory have shown that extracts derived from grape seeds causes an endothelium dependent relaxation in rings of the rabbit aorta that is mediated by nitric oxide. This process is initiated by phosphorylation of nitric oxide synthase through the PI3K/Akt pathway. Inhibition of this pathway also abolishes the endothelium dependent relaxation and up-regulates nitric oxide synthase in human umbilical vein endothelial cells [9]. In humans, the extract was also found to lower blood pressure in patients diagnosed with the metabolic syndrome [10]. A similar effect has also been demonstrated with a freeze dried product of grapes in people with the metabolic syndrome [11].

The investigation reported here was undertaken to test the hypothesis that a well characterized extract of grape seeds lowered blood pressure in subjects with pre-hypertension. The trial was a single center, double blind, placebo controlled, parallel arm study which lasted 8 weeks. The study was approved by the Internal Review Board of the University of California.

METHODS

The study was conducted on a convenience sample of 66 adults (age 25-80 years) who were screened for pre-hypertension. Those with average day time blood pressures which met the JNC 7 criteria for pre-hypertension (systolic blood pressures between 120 and 139 mmHg or diastolic blood pressures between 80 and 89 mmHg) were enrolled in the trial after obtaining written consent. The exclusion criteria were as follows: smokers (abstinence for < 1 year), clinical

evidence of coronary artery, pulmonary, gastro intestinal or renal disease, consumption of prescription medications and vitamin preparations.

After baseline biochemical and hematological parameters were measured, all subjects commenced a two-week placebo run-in period. During this period they were fitted with an ambulatory blood pressure measuring system to confirm the diagnosis of pre-hypertension. (Model SE-25S; Sein Electronics, Koyang, South Korea). This system has been evaluated using a protocol approved by the British Hypertension Society (www.tiba.medical.com). It was programmed to record the blood pressure every hour for 12 hours after waking up. At the end of two weeks, the subjects had a second ambulatory blood pressure measurement (12 hour) and were randomized subsequently to receive a capsule containing either a placebo (Maltodextrin) or a grape seed extract (300 mg) daily. The grape seed extract used in this study was Meganatural BP ® (Polyphenolics Inc., Madera, California). The subjects were advised to maintain their usual level of activity and diet. The latter was monitored by examining a 4-day food diary which was completed at the start and at the end of the study. After a further 8 weeks, a final ambulatory blood pressure was recorded and blood was drawn for measurement of biochemical and hematological parameters. In each instance, the average of 12 values was taken as the mean day time blood pressure.

The distribution of phenolic compounds in the grape seed extract is shown in Table **1**. The ORAC value of the compound was 16,810 µmol Trolox equivalents/g. The average degree of polymerization is 2.3. (These details are archived with reference [9]). In a previous study, administration of this grape seed extract (300 mg) (n=5) resulted in a 10-fold increase in plasma catechin levels from a baseline value of 2.0 ± 4 after 90 minutes. There were no significant changes in subjects given placebo capsules [10].

Fasting blood samples were collected for the following measurements at the start of the study and at the end: hemoglobin, white cell count with differential, serum lipids, chemistry panel, blood glucose, plasma insulin, and oxidized low-density lipoprotein (Ox-LDL).The Ox-LDL concentration in plasma was measured using an mAb-4E6–based enzyme-linked Immunosorbent assay (Mercodia, Uppsala, Sweden). The analysis was undertaken by Shiel Laboratories, New York.

Statistical Analysis

The primary endpoints were the mean day-time systolic and diastolic blood pressures. Secondary endpoints were the changes in serum lipids and oxidized LDL. Baseline values in the 2 groups were compared using a t test. A p value of 0.05 with an associated power of 0.08 was taken to indicate statistical significance.

RESULTS

Sixty six subjects were screened for the study and 34 met the criteria for pre-hypertension. Two refused to participate in the trial and remaining 32 were randomized. The baseline clinical data are given in Table **2**. There were no significant differences in the baseline parameters in these subjects.

At the end of 8 weeks both systolic and diastolic blood pressures in the group receiving GSE were significantly lower than those in the placebo group. These findings are summarized in Table **2**. There were also no changes in body weight, blood counts, serum electrolytes and chemistry and glucose values during the course of the study.

There were also no changes in the serum total, LDL and HDL cholesterol values in both groups. An interim analysis was performed on the oxidized LDL values after 8 subjects in each group had completed the study.

Table 1: Composition of the Grape Seed Extract (n = 8). Original Data Archived with Ref [9] http://www.clinsci.org/cs/114/cs1140331add.htm

Total Phenol content (gallic acid equivalents (g/100g)	93.9± 0.9
Epicatechin gallate terminal units (%)	0%
Epicatechin gallate extension units (%)	5.7± 0.6
Monomers (%) *	9.1± 1.2
Oligomers (%) *	68.7± 1.2
Polymers (%) *	22.3± 0.6
Catechin and epicatechin by weight (%)	9.9± 0.6

*Determined by reverse-phase HPLC using peak area.

Table 2: Baseline Clinical Data

	Placebo	GSE
Age (yr)	54±3	50±2.5
Male/female	6/10	9/7
Total cholesterol (mg/dl)	204±9	200±10
LDL (mg/dl)	134±9	128±9
HDL (mg/dl)	48 ±3	55±4
Triglycerides (mg/dl)	100±12	146±18
Oxidized LDL (mU/l) (n=8)	43.3±3	41.2±3

It was found that the baseline values were similar in both groups (Table **1**) and the there were no significant changes after two months in either group. These interim measurements were done without compromising the blinded status of the study. No additional measurements of oxidized LDL were undertaken on the other subjects.

DISCUSSION

This study was undertaken to test the hypothesis that polyphenolic compounds found in grape seed lowers blood pressure in people with pre-hypertension. It was a follow up to a previous study which showed that these compounds lowered blood pressure in people who met the diagnostic criteria for the metabolic syndrome. Both these conditions affect nearly half the adult population of the United States and the current recommendations of the National Cholesterol Education Program [12] and the JNC-7 [2] are that the majority of these patients should be managed by encouraging them to undertake lifestyle changes which address weight management, physical activity, reducing the intake of salt and dietary/nutritional changes. The latter includes the consumption of two cups of fruit and 2½ cups of vegetables per day for a reference 2,000-calorie intake. It has been suggested that fruits and vegetables, particularly those with higher polyphenolic content such as grapes, strawberry, blueberry and pomegranate, influence multiple biological mechanisms which could have favorable effects on human health due to their ability to modulate oxidative and inflammatory stress in peripheral tissues [13-16].

Grape seeds contain approximately 3000 mg of phenols/kg of fresh weight made up principally of monomeric flavan-3-ols (which includes among other compounds (+) catechin and (-) epicatechin) , oligomeric proanthocyanidins and polymeric condensed tannins [17]. The extract used in the present study contained significant quantities of oligomers and no terminal gallate units (see methods). This particular extract has been shown to produces an endothelium dependent relaxation in rings of the rabbit aorta in-vitro [9]. The endothelium dependent relaxation evoked by the extract is mediated by the activation of the PI3K/Akt signalling pathway, resulting in the phosphorylation of eNOS through a redoxsensitive mechanism [18]. Removal of the antioxidant activity from the extract by methylation of the hydroxy groups abolished the endothelium dependent relaxation induced by the grape seed extract [9].

Based upon this evidence, a small placebo controlled clinical trial was undertaken in patients with the metabolic syndrome to determine whether this grape seed extract lowered the blood pressure. This study showed that the extract when administered orally

Table 3: Changes in Blood Pressure (mmHg)

	GRAPE SEED EXTRACT 300 mg/day (n = 16)		PLACEBO (n = 16)	
	SBP	DBP	SBP	DBP
Start	133 ± 2	79 ± 2	132 ± 2	79 ± 2
2 months	125 ± 2	74 ± 2	133 ± 2	82 ± 2
p	<0.001	<0.003	0.8	0.03
Power at p<05	>0.9	>0.85	NS	0.5

at a dose of 300 mg daily resulted in a significant reduction in blood pressure. The extract also appeared to reduce the concentration of oxidized LDL particularly when the baseline values were greater than 60 mg/l. In addition the plasma also showed evidence of absorption of polyphenolic compounds. A recently reported study by Barona *et al.* [11] showed that consumption of a freeze dried powder of grape products also resulted in a reduction of blood pressure in patients who had the metabolic syndrome. The subjects consumed sufficient quantities of the powder to yield approximately 266 mg of phenols.

In the present study we examined the effect of the extract on blood pressure in people with pre-hypertension as defined by the Joint National Committee. In this placebo controlled study, there was a significant reduction in both systolic and diastolic blood pressures. There was no effect on oxidized LDL. However, unlike in the patients with the metabolic syndrome who participated in the previous study [10], all the subjects in the in the present study had plasma oxidized LDL values that were less than 50 mU/l.

LIMITATIONS

Several studies have shown that the consumption of appropriate quantities of fruit and vegetables in the United States falls far short of current recommendation (e.g. [19]). Nutraceutical supplementation could provide a means of addressing some of the health problems that stem from an inappropriate diet in the short and medium term such as hypertension and obesity. The studies described in this paper are essentially small trials that attempt to prove the concept that polyphenolic compounds present in grape seed are prototypes of biologically active compounds commonly found in fruits and vegetable which could form the non-pharmaceutical basis for managing pre-hypertension. It is recognized that larger placebo controlled long-term trials (conducted extending over several years) are required to determine whether these compounds reduce the number of people transitioning from pre-hypertension to overt hypertension.

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Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract

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ABSTRACT

GSEs (grape seed extracts) which contain polyphenolic compounds cause an endothelium-dependent relaxation of blood vessels. The aim of the present study was to examine the mechanisms involved in this response. A well-characterized GSE was applied to rabbit aortic rings suspended in organ baths containing Krebs–Henseleit buffer maintained at 37 °C. In aortic rings pre-contacted with noradrenaline (norepinephrine), the extract produced a dose-dependent relaxation. The maximum relaxations elicited by the extract (71.9 ± 1.0 %) were similar to those elicited by acetylcholine (64.2 ± 1.5 %) ($n = 12$ for each). As expected, the relaxations were abolished by removal of the endothelium and by prior incubation with L-NAME (N^G-nitro-L-arginine methyl ester), confirming the essential role of eNOS (endothelial NO synthase) in the response. The responses to the GSE were also abolished by incubation with wortmannin and LY294002, which are inhibitors of PI3K (phosphoinositide 3-kinase). These compounds had no effect on the responses to acetylcholine. Using immunoblotting, we also demonstrated that the GSE induced the phosphorylation of both Akt and eNOS in HUVECs (human umbilical vein endothelial cells). Finally, the extract was modified by methylation of the hydroxy groups in the polyphenolic groups and was applied to the aortic rings. The modified extract failed to cause a relaxation. Taken together, these findings suggest that the endothelium-dependent relaxation induced by the GSE was mediated by activation of the PI3K/Akt signalling pathway through a redox-sensitive mechanism, resulting in phosphorylation of eNOS.

INTRODUCTION

There is evidence that a diet rich in vegetables and fruit has a beneficial effect on blood pressure. This effect has been attributed to phenolic compounds present in the plants. These compounds have also been shown to influence endothelial function in a variety of experimental situations [1–3]. In humans, extracts of fruits and vegetables have been shown to enhance flow-mediated vasodilation in the brachial artery [4].

Of all of the phenolic products, those derived from grapes appear to have received the most attention, possibly because of their involvement with the French paradox [5,6]. Grapes and grape products derived from the skin, seeds, pulp and stem are good sources of polyphenolic compounds; however, it has been found that >70 % of polyphenolic compound are concentrated in the seeds [7]. GSEs (grape seed extracts) cause an EDR (endothelium-dependent relaxation) of aortic rings *in vitro* (for example, [2,8,9]). Similarly, these extracts

Key words: bioactive phenolic, endothelium-dependent relaxation, endothelial nitric oxide synthase (eNOS), grape seed extract, phosphoinositide 3-kinase (PI3K), reactive oxygen species (ROS).

Abbreviations: EDR, endothelium-dependent relaxation; GSE, grape seed extract; HUVEC, human umbilical vein endothelial cell; KH buffer, Krebs–Henseleit buffer; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, NO synthase; eNOS, endothelial NOS; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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have also been shown to activate eNOS [endothelial NOS (NO synthase)] [2,10] and up-regulate eNOS in cultured endothelial cells [11].

The mechanism mediating this response to GSEs has not been established with certainty. Grape juice [12] and extracts of red wine [13] have been shown to cause EDR, which was abolished by blocking the PI3K (phosphoinositide 3-kinase)/Akt pathway. In the present study, we have examined the effect of a well-characterized GSE, which has been shown previously to cause EDR in guinea pig aortic rings [14] and reduce blood pressure in humans [15], on the PI3K/Akt signalling pathway and phosphorylation of eNOS. The studies were undertaken on both rabbit aortic rings and HUVECs (human umbilical vein endothelial cells).

MATERIALS AND METHODS

Study design and procedures

This study was approved by Animal Use and Care Administrative Advisory Committee, University of California, Davis, CA, U.S.A. Male New Zealand rabbits, weighing 3–3.5 kg, were sedated by intramuscular injection of acepromazine. After 5 min, a lethal dose of sodium pentobarbitone (50 mg/kg of body weight; Abbott Laboratory) was administrated through the lateral ear vein. A thoracotomy was performed and the descending thoracic aorta was excised carefully. The aorta was flushed twice with fresh ice-cold KH (Krebs–Henseleit) buffer (118 mmol/l NaCl, 5.4 mmol/l KCl, 1.2 mmol/l, $MgCl_2$ 2.5 mmol/l $CaCl_2$, 22 mmol/l $NaHCO_3$, 1.2 mmol/l NaH_2PO_4 and 10.1 mmol/l glucose; using Sigma analytical grade reagents) and placed in a dissecting tray filled with the same buffer. All surrounding connective tissues and fat were removed carefully.

The GSE used in the present study is a water extract prepared by Polyphenolics Inc (Meganatural-BP®; patent pending). The extract is made up of polymers of catechin and has an average degree of polymerization of 2.3. The extract was dissolved in KH buffer, and the concentrations of the solution were based on a nominal M_r of 1000. The phenol content of the GSE solution (1 mg/ml) was measured using the Folin–Ciocalteu assay and was found to be 39.2 ± 0.65 mmol/l gallic acid units ($n = 5$). The characterization of the extract is given in Supplementary material available at http://www.clinsci.org/cs/114/cs1140331add.htm.

Measurement of EDR

EDR was assessed as described previously [16]. Briefly, the aorta was segmented into rings (5 mm in length) which were mounted between two tungsten wire triangles. One triangle was attached to a strain-gauge transducer and the other to the bottom of an organ bath (20 ml) containing KH buffer maintained at 37 °C and oxygenated with a mixture of 95 % O_2/5 % CO_2. A pre-load of 8 g was applied to the rings, and the tissues were allowed to equilibrate for 60 min. The transducer was connected to a pen recorder (Gould-2400S recorder), and the changes in tensions were monitored using a Windaq computer program (2003 version; Dataq Instruments).

After equilibration for 60 min at a pre-load of 8 g, the aortic rings were pre-contracted with 10 μmol/l noradrenaline (norepinephrine; Sigma). Acetylcholine (Sigma) was added in an incremental manner to achieve bath concentrations from 0.1–10 μmol/l to obtain dose–response curves for EDR. The relaxations were expressed as a percentage of the contraction induced by noradrenaline.

GSE-induced EDR

After demonstrating EDR evoked by acetylcholine, the rings were treated with increasing concentrations of the GSE following pre-contraction with noradrenaline. In additional experiments, the effect of removing the endothelium on relaxation evoked by acetylcholine and the GSE were examined to establish the endothelium-dependent nature of the relaxation. In these experiments, after demonstrating the absence of relaxation, the rings were treated with SNP (sodium nitroprusside; Sigma) to establish the ability of the aortic smooth muscle to relax. As a further control, the effect of incubation with L-NAME (N^G-nitro-L-arginine methyl ester; bath concentration, 1 mmol/l; Sigma), a competitive inhibitor of NOS, was examined to demonstrate the involvement of NOS in the relaxation of the rings.

Effect of blocking the PI3K/Akt pathway on EDR induced by the GSE

Previous studies have shown that the EDR evoked by polyphenolic compounds derived from grapes was abolished by inhibitors of the PI3K/Akt pathway [13,17], In the present study, the effect of the GSE was examined after incubating the aortic rings with wortmannin (30 nmol/l; Sigma) and LY294002 (30 μmol/l; Sigma) in KH buffer. Both wortmannin and LY294002 are potent and specific PI3K inhibitors. In testing the effect of each inhibitor, three aortic rings were tested simultaneously according to the sequence shown in Table 1. This protocol was based on a previous finding that prior exposure to the GSE and other phenolic compounds (e.g. cocoa) attenuated the effect of subsequent exposure [2,10]. Thus it is not possible to expose a ring to the same extract twice, before and after exposure to the inhibitor, and obtain meaningful data.

Step 1 was done to establish responsiveness of the rings to a standard concentration of acetylcholine, step 2 provided a baseline dose–response curve to acetylcholine, and steps 3 and 4 established the effect of the inhibitors.

Ring 1 was used to examine the effect of the extract after incubation with the inhibitor, and ring 2 was used to demonstrate the response to the extract without prior

Table 1 Protocol for testing the effect of the PI3K inhibitors on EDR induced by the GSE

Step	Ring 1	Ring 2	Ring 3
1	Acetylcholine (10 μmol/l)	Acetylcholine (10 μmol/l)	Acetylcholine (10 μmol/l)
2	Dose–response curve with acetylcholine	Dose–response curve with acetylcholine	Dose–response curve with acetylcholine
3	Incubate with PI3K inhibitor for 30 min	No incubation, KH buffer alone	Incubate with PI3K inhibitor for 30 min
4	Dose–response curve with the GSE	Dose–response curve with the GSE	Dose–response curve with acetylcholine

exposure to the inhibitor. It also showed that prior exposure to acetylcholine did not influence the response to the extract (i.e. the maximal responses were similar). Ring 3 was used to demonstrate that the response to acetylcholine was unaltered with time (time control) and that exposure to the blocker did not affect the ability of eNOS to be activated by acetylcholine. This protocol avoided the application of the extract twice in succession to a ring.

Effect of wortmannin and LY294002 on phosphorylation of eNOS and Akt

HUVECs were grown in EGM-2 medium(Cambrex) with 10 % (v/v) fetal bovine serum. Cells were grown to confluence (approx. 90 %) and starved for 6 h in serum-free medium before the cells were treated with the GSE (10 μmol/l). Some wells were treated with LY294002 (30 μmol/l) or wortmannin (30 nmol/l) for 30 min before exposure to the GSE. Cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 10 min. The reaction was stopped by adding ice-cold PBS, washed twice with PBS and cell lysates were prepared in RIPA buffer [20 mmol/l Tris/HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 % Nonidet P40, 1 % sodium deoxycholate, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l sodium orthovanadate and 1 μg/ml leupeptin]. Total proteins (30 μg) were separated by SDS/PAGE [7.5 % (w/v) polyacrylamide gels] and were transferred electrophoretically on to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with blocking buffer containing 5 % (w/v) non-fat milk in TBS-T (Tris-buffered saline containing 0.1 % Tween 20) for 1 h. Phosphorylated Akt (at Ser^{473}), phosphorylated eNOS (at Ser^{1177}), Akt and eNOS were detected after the membranes were incubated with the respective primary antibodies {rabbit anti-[phospho-eNOS (Ser^{1177})], anti-eNOS, anti-[phospho-Akt (Ser^{473})] and anti-Akt; 1:1000 dilution; Cell Signaling Technology} overnight at 4 °C. Membranes were washed three times (10 min each) and incubated with the secondary antibody [HRP (horseradish peroxidase)-labelled anti-(rabbit IgG); 1:20 000 dilution; Cell Signaling Technology] at room temperature (25 °C) for 60 min. Membranes were washed three times again (10 min each) and the specific protein bands were visualized using ECL® (Amersham Biosciences). All four proteins were detected on the same blot, and the membranes were washed with stripping buffer (Pierce Biotechnology) for 30 min in 37 °C before being incubated with the next primary antibody.

Effect of methylated GSE on EDR

An additional series of experiments were undertaken to study the effect of methylated GSE on rings of rabbit aorta. In each experiment, two rings were prepared as described above. Ring 1 was exposed to acetylcholine (10 μmol/l) and the GSE (100 μmol/l), and ring 2 was treated sequentially with acetylcholine (10 μmol/l), methylated GSE and the GSE (both 100 μmol/l). The methylation procedure is outlined in the Supplementary material available at http://www.clinsci.org/cs/114/cs1140331add.htm.

Statistical analysis

Group data are expressed as means ± S.E.M. Comparisons between groups were compared using a paired Student's *t* test or ANOVA depending on the number of groups being examined. Dose–response curves were compared using repeated measures ANOVA. Data were analysed using Sigma Stat (version 3, 2003) statistical software. Statistical significance among treatments was determined as $P < 0.05$.

RESULTS

Effect of the GSE on EDR

The GSE produced a dose-dependent relaxation of the aortic rings. The maximum relaxations observed were similar to those produced by acetylcholine (Figure 1). Removal of the endothelium abolished the responses evoked by acetylcholine and the GSE, confirming the obligatory role of the endothelium. Incubation with L-NAME, a competitive eNOS inhibitor, also abolished the relaxation responses to acetylcholine and the GSE. However, thereafter the rings remained responsive to SNP, which is a non-endothelium-dependent relaxant of smooth muscle (Figure 2). The maximum relaxations observed in the rings under the different conditions are summarized in Table 2. These results confirmed that the GSE causes EDR in rings of rabbit aorta.

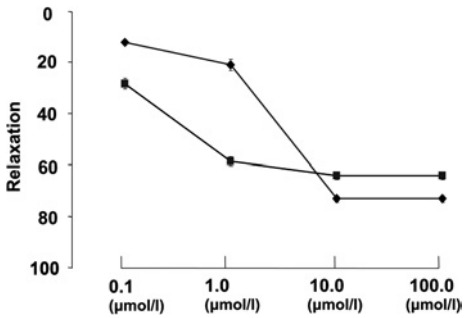

Figure 1 Dose-dependent relaxation of aortic rings induced by acetylcholine and the GSE

Dose–response curves relating relaxation (as a percentage of contraction to 10 μmol/l noradrenaline) and concentration of the agonists in the organ bath. Dose–dependent relaxations were evoked by acetylcholine (◆) and the GSE (■). Values are means ± S.E.M. ($n = 12$).

Effect of inhibitors of the PI3K/Akt pathway

Incubation of aortic rings which had been previously shown to be responsive to acetylcholine with wortmannin or LY294002 significantly attenuated the relaxation induced by the GSE. The responses evoked by acetylcholine were unaffected. The sequence of treatments described in the Materials and methods section were used in these experiments. An example of an experiment with each blocker is shown in Figure 3, showing that the GSE-induced dose-dependent EDR was significantly attenuated in rings exposed previously to a PI3K inhibitor. The responses induced by the highest concentration of the GSE in these experiments are shown in Figure 4. It was also confirmed that acetylcholine-induced EDR was unaffected by PI3K inhibitors. Therefore it is apparent that prior exposure to a PI3K inhibitor attenuated EDR evoked by the GSE, suggesting that EDR induced by the GSE is mediated by the activation of the PI3K/Akt pathway.

Table 2 Summary of the maximum relaxations observed in the aortic rings

Values are means ± S.E.M. of maximum percentage relaxation evoked by different treatments ($n = 6$). Values with different superscripts are significantly different, as determined by ANOVA ($P < 0.05$).

Agent	Maximum relaxation (%)
Acetylcholine (10 μmol/l)	64.2 ± 1.5^{a}
GSE (100 μmol/l)	71.9 ± 1.0^{b}
L-NAME (1 mmol/l) + GSE (100 μmol/l)	6.3 ± 1.0^{c}
SNP (10 μmol/l)	81.4 ± 1.6^{d}

GSE induces the phosphorylation of Akt and eNOS in HUVECs

EDR is caused by NO produced by the phosphorylation of eNOS. Therefore we investigated whether the GSE induced the phosphorylation of Akt (on Ser^{473}) and eNOS (on Ser^{1177}) *in vitro* in HUVECs. The GSE-induced phosphorylation of Akt and eNOS was shown by immunoblotting. Prior exposure to the PI3K inhibitor LY294002 abolished the phosphorylation of Akt and eNOS in HUVECs (Figure 5). These results suggested that the GSE phosphorylates eNOS through a PI3K/Akt pathway.

Effect of methylation of the GSE on EDR

It was found that methylated GSE failed to produce an ERD in the aortic rings. Subsequent exposure to the GSE (100 μmol/l) produced a significant relaxation, which was similar to that evoked by acetylcholine (10 μmol/l) (Figure 6).

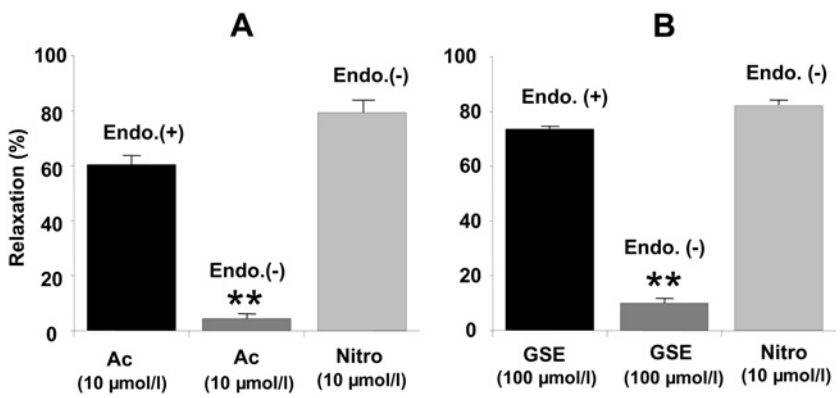

Figure 2 Effect of removal of endothelium on the maximum relaxation

(A) Responses induced by acetylcholine (Ac). (B) Responses induced by the GSE. Removal of endothelium abolished the responses elicited by acetylcholine and the GSE. The rings remained responsive to SNP (Nitro), which is a non-endothelium-dependent relaxant of smooth muscle. Values are means ± S.E.M. ($n = 4$). $^{**}P < 0.01$ compared with the treatment with the endothelium present and SNP with the endothelium removed. Endo.(+), endothelium present; Endo.(−), endothelium removed.

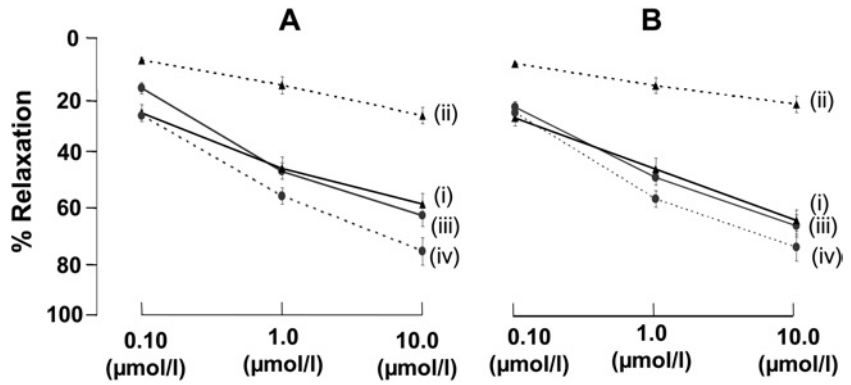

Figure 3 Dose–response curves evoked by the GSE after incubation with (A) wortmannin and (B) LY294002

Treatment with the agonists and inhibitors are described in the Materials and methods section and Table 1. (A) Initial response to acetylcholine [curve (i)], response to the GSE after incubation with wortmannin (30 nmol/l) for 30 min [curve (ii)], initial response to acetylcholine [curve (iii)], and response to GSE without prior incubation with wortmannin [curve (iv)]. Dose–response curves (i) and (ii) were generated from ring 1, and curves (iii) and (iv) were generated from ring 2. (B) Initial response to acetylcholine [curve (i)], response to the GSE after incubation with LY294002 (30 μmol/l) for 30 min [curve (ii)], initial response to acetylcholine [curve (iii)], and response to the GSE without incubation with LY294002 [curve (iv)]. Dose–response curves (i) and (ii) were generated from ring 1, and curves (iii) and (iv) were generated from ring 2. Curve (ii) is different from other three in both (A) and (B). Values are means $\pm$ S.E.M. ($n = 4$) in both A and B. Results from ring 3 are not shown.

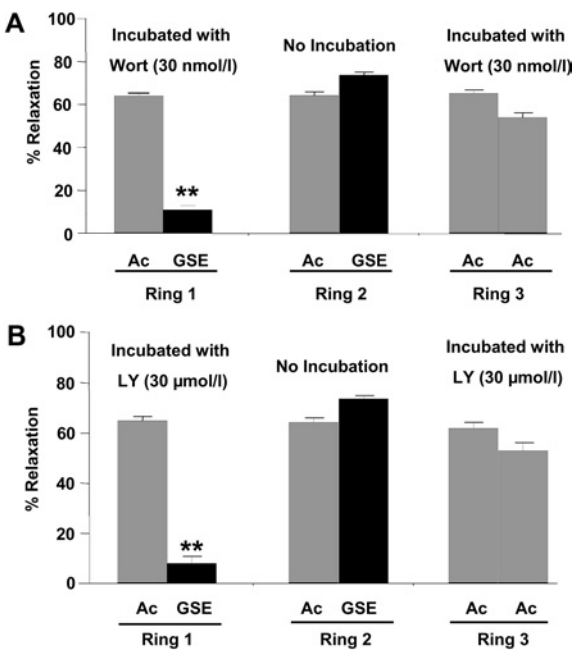

Figure 4 Effect of PI3K inhibitors on the maximum relaxation produced by GSE

(A) All three rings responded to acetylcholine initially. Ring 1, which was incubated with wortmannin (30 nmol/l for 30 min) and tested with the GSE, had a significantly attenuated relaxation (**$P < 0.01$ compared with GSE alone). Ring 2, which was not incubated with wortmannin, had a similar relaxation with the GSE. Ring 3, which was also incubated with wortmannin, had no significant change in the responses to acetylcholine. (B) Similar findings were observed with LY294002 (30 μmol/l) for the effect induced by the GSE. The relaxation evoked by the GSE was significantly decreased (**$P < 0.01$ compared with GSE alone). All values are means $\pm$ S.E.M. ($n = 4$).

DISCUSSION

The present study has shown that the GSE used produced EDR in the rabbit aorta, which was significantly attenuated by prior incubation with the PI3K inhibitors wortmannin and LY294002. In these respects, the response is similar to that evoked by other derivatives of grapes which have been investigated extensively [12,13,18]. The novel aspects of the present study are the following: (i) the concurrent phosphorylation of both Akt and eNOS; (ii) modifying the antioxidant activity of the extract by methylation removed the ability to cause EDR; and (iii) we have used a compound that is very high in phenols (>90%), unlike GSEs used in other studies. Overall, the GSE used in the present study has been analysed in much greater detail than ones used by other investigators (see Supplementary material available at http://www.clinsci.org/cs/114/cs1140331add.htm).

Potential mechanism of action

Akt is a serine/threonine protein kinase that is recruited to the (endothelial) membrane by binding to PI3K-produced phosphoinositides. At the membrane, Akt is phosphorylated and activates eNOS (by phosphorylation at Ser^{1177} in humans), leading to the production of NO [17]. It has also been shown that the production of NO in response to fluid shear stress is controlled by Akt-dependent phosphorylation of eNOS [19]. However, recent studies performed in cell culture have established that polyphenolic compounds in red wine also affect the level of phosphorylation of Akt in a PI3K-dependent manner, which in turn phosphorylates

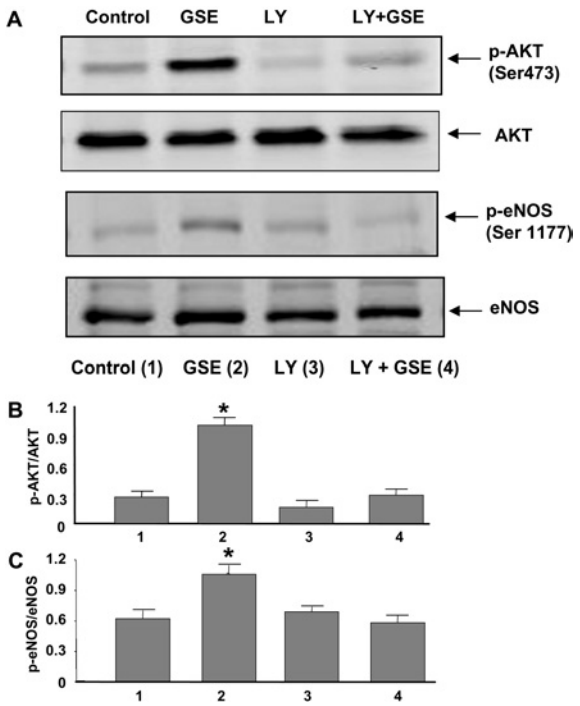

Figure 5 Effect of the GSE on eNOS and Akt phosphorylation

(A) HUVECs were treated with vehicle (control; lane 1), GSE (lane 2), LY294002 alone (lane 3) and GSE plus LY294002 (LY+GSE). In the controls, low levels of phosphorylated Akt and eNOS were observed, which were increased after incubation with GSE (lane 2). LY294002 alone (lane 3) had no effect on phosphorylation compared with the controls, but inhibited GSE-induced phosphorylation of Akt and eNOS when incubated with GSE (lane 4). (B and C) Quantification of the immunoblots of phosphorylated Akt (p-Akt) (B) and phosphorylated eNOS (p-eNOS) (C) using densitometry ($n = 4$). The results are ratios of the phosphorylated and non-phosphorylated form of each enzyme. $^*P < 0.05$ compared with the control.

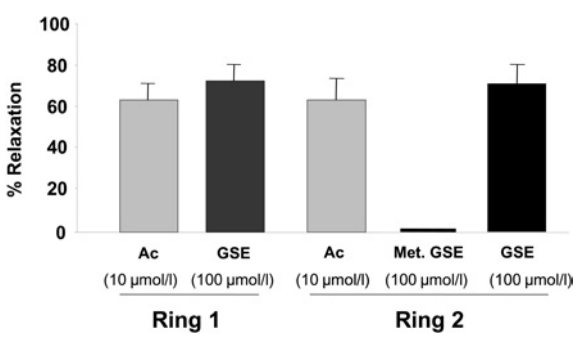

Figure 6 Effect of methylation of the GSE on EDR

The responses induced by acetylcholine (Ac), the GSE and methylated GSE (Met. GSE) are shown. Ring 1 had acetylcholine and the GSE applied in sequence, and Ring 2 had acetylcholine, methylated GSE and the GSE applied in sequence. No relaxation was evoked by methylated GSE ($n = 4$).

eNOS, resulting in an increased formation of NO [13]. PI3K, which is a redox-sensitive protein kinase, appears to be activated by the redox sensitivity of polyphenols,

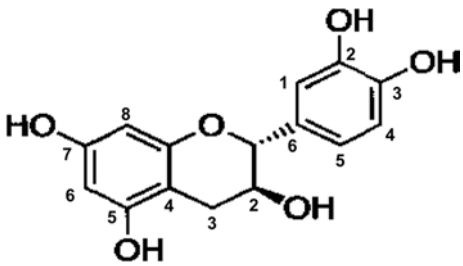

Figure 7 Basic structure of flavanoids

Proanthocyanidins are polymeric phenolic compounds characterized by a flavanoid with the basic three-ring structure.

leading to the production of NO. It has also been shown that, in endothelial cells, phosphorylation induced by polyphenols occurs on Ser^{1177} of eNOS and dephosphorylation at Thr^{495} within a few minutes of exposure. These changes in the phosphorylation level of eNOS were maintained for at least 30 min.

The GSE used in the present study is a relatively 'pure' one, with phenolic compounds forming 93 % of its constituents. These compounds are mostly proanthocyanidins which occur as mixtures of oligomers and polymers of catechin and epicatechin (see Supplementary material available at http://www.clinsci.org/cs/114/cs1140331add.htm). Some of the larger polymeric compounds have the capacity to complex with proteins to form tannins. Plant tannins are divided into hydrolysable and condensed forms. The former contains gallic acid and a dimeric condensation product (hexahydroxydiphenic acid) that is esterified to a polyol such as glucose. The condensed tannins are high-molecular-mass oligomers and polymers of the monomeric unit flavanol-3-ol and their gallic acid esters. The monomeric units themselves are formed through oxidative condensation by carbon–carbon bonds, normally between carbon-4 of the heterocycle carbon ring and carbon-8 of the adjacent units (Figure 7). The GSE used in the present study consisted mainly of dimers and trimers (see Supplementary material available at http://www.clinsci.org/cs/114/cs1140331add.htm) and was devoid of gallic acid residues.

Polyphenolic compounds are generally considered to be antioxidants [20,21]; however, under certain circumstances, they have pro-oxidant properties attributable to the hydroxy groups in the phenolic rings. For instance, treatment of cell cultures with polyphenolic compounds significantly increased the production of ROS (reactive oxygen species) such as H_2O_2 [22,23]. It has been proposed that H_2O_2 is generated by auto-oxidation of hydroxy groups present in phenolic compounds (see Figure 1 in [23]), which subsequently activate PI3K [13,23]. Ndaye et al. [13] have shown that removal of hydroxyl radicals derived from H_2O_2

by enhancing endogenous SOD (superoxide dismutase) abolished the EDR produced by GSEs. In the present study, we have demonstrated that the removal of the hydroxy groups from the GSE by prior methylation also abolished EDR, thus supporting the important role of the hydroxy groups in producing EDR.

Conclusions

In the present study, we provide evidence to suggest that EDR evoked by the GSE is mediated by the activation of the PI3K/Akt signalling pathway, resulting in the phosphorylation of eNOS. Previous studies have suggested that GSEs activate PI3K and downstream signalling via Akt and activate eNOS through a redox-sensitive mechanism [13]. Furthermore, we found that removal of the antioxidant activity from the GSE by methylation of the hydroxy groups abolished the EDR induced by GSEs. These results support the suggestion that ROS produced by GSEs can activate eNOS to produce NO and cause vasodilation.

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