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Vasoactive Properties of Keratin-Derived Compounds

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Abstract

Objective—Keratin proteins have been utilized as biomaterials for decades and are currently under investigation for a variety of tissue regeneration and trauma applications. It has been suggested that certain keratins may have the capacity to act as a colloid in fluid resuscitation applications, providing viscosity and oncotic properties that may be beneficial during acute ischemic events. Oxidized keratin derivatives, also known as keratoses, show good blood and cardiovascular compatibility and thus are the subject of this study.

Methods—The effects of Keratose compounds will be assessed using a topload intravenous infusion model and observation of changes in the microvasculature of the cremaster muscle of rats.

Results—Keratose resuscitation fluid (KRF) administration resulted in significant vasodilation in the cremaster muscle. This effect was blocked with pretreatment of L-NA to inhibit nitric oxide. Another keratin fraction, alpha keratose, which is the primary viscous compound, was not found to induce vasodilation.

Conclusions—The apparent mechanism of vasodilation was found to be nitric oxide- mediated and isolated to a particular purified fraction, the keratin associated proteins (KAP).

Keywords

keratin; biomaterial; keratin-associated protein; KAP; vasodilation; resuscitation; ischemia

Introduction

Human hair keratins can be classified into three broad groups, alpha- and beta-keratins, as well as the keratin associated proteins or KAP fraction (often referred to as gamma-keratin). Alpha-keratins are the main structural component in hair fibers, have a relatively high molecular weight and low sulfur content, and are the most abundant [18]. KAP are globular, lower in molecular weight, higher in sulfur content, and act as a matrix that holds the cortical super structure together [16]. The beta-keratins are primarily protective and constitute most of the cuticle [15]. They are significantly more difficult to extract and do not form useful reconstituted structures.

The keratins found in hair fibers are characterized by generally high cysteine content and disulfide crosslinking. In order to extract useful keratins from end-cut human hair fibers, these disulfide crosslinks must be broken without appreciable disruption of amide bonds, then the strong hydrogen bonding also present in keratinous tissues must be overcome. If hydrogen peroxide or peracetic acid oxidant is used for this disulfide bond scission, cysteine side groups are converted to sulfonic acid and do not readily react with other materials. These keratin derivatives are referred to as “keratose”, which has been investigated as a biomaterial for a number of medical applications and shown to have interesting properties including excellent cell and tissue compatibility [5]. Keratoses can be formulated as a biocompatible fluid with various viscosities and have previously been identified as potential constituents in a resuscitation fluid [21]. In a preliminary experiment described within the patent, Widra showed that intravenous injection of keratose- containing fluid did not induce untoward effects in dogs [21]. The patent states that after 25% blood volume hemorrhage and keratin-containing fluid replacement, the animal showed no signs of anaphylaxis, toxicity, red cell damage, or elevated counts of eosinophils, basophils, lymphocytes, or monocytes.

High viscosity fluids may help release potent vasodilators such as nitric oxide (NO) and prostaglandins when infused intravenously (i.v.). The release of these mediators is induced through vascular wall shear stress [3, 6]. The mechanism for NO release has been linked to mechanoreceptors within endothelial cells that are stimulated by shear stress and appears to be linked to a G protein-coupled receptor mechanism [10]. Furthermore, endothelial cells exposed to laminar shear stress have demonstrated enhanced eNOS transcription factors such as eNOS 3' polyadenylation resulting in eNOS expression [13]. Keratose also possesses high tonicity, which may induce net transport of water from the extravascular space into the vascular system. Small molecules can be eliminated from the final product through dialysis in order to avoid leakage of these compounds out of the vasculature. Keratoses are also compatible with the cells and tissues of the cardiovascular system. While the full therapeutic potential of keratins remains to be determined, several clinical pathologies may benefit from treatment with a fluid having these characteristics. We hypothesized that hyperviscous keratose fluids would provide the most resuscitative benefit, simply due to their mechanical effects and hygroscopic nature, and endeavored to study their behavior in the microcirculation. Keratose-based fluids formulated at different viscosities were compared to another high viscosity compound, Hetastarch 6% in saline solution. Surprisingly, the keratins were observed to have vasoactive properties, so a potential physiologic mechanism and the source of keratose-induced vasodilation was also investigated in this study.

Methods

Keratose was prepared as previously described [20]. Briefly, human hair was obtained from a commercial vendor and treated with peracetic acid to break protein disulfide crosslinks. Residual oxidant was removed by rinsing the retted hair and soluble keratins extracted into

excess tris base and deionized (DI) water. The extracted keratose solution was dialyzed with a 30 kiloDalton NLMWCO cartridge in order to eliminate small molecules from the final product, thus avoiding leakage of these compounds out of the vasculature. The dialyzate was concentrated, neutralized, lyophilized, and sterilized by gamma irradiation (1M Rad). Resuscitation fluids were prepared by aseptic reconstitution of the keratose powder with phosphate buffered saline at the desired concentration. Viscosity was determined with a DV-II+ Pro cone and plate viscometer at 37 °C and shear rate of 275 sec⁻¹ (Brookfield Engineering Laboratories, Inc., Middleboro MA).

In order to assess the native molecular weight of the keratin compounds isolated by the process described above, size exclusion chromatography [19] was performed. Samples were dissolved at 1-2 weight/volume percent (depending on viscosity) in 10 mM HEPES + 130 mM sodium chloride, pH 7.4 buffer. A Biologic LP chromatography system (Bio-Rad Laboratories, Hercules CA) using a 1×30cm Sephacryl S-300 column (GE Healthcare, Piscataway NJ) running at 0.17 mL/min was injected with approximately 800 L of sample. Detection of proteins was at 250 nm. Standards at 340, 230, 128, and 64KDa molecular weight were analyzed under identical conditions and molecular weights of the keratose samples calculated from the Log(10) molecular weight-peak elution time relationship.

Under a protocol approved by the Wake Forest Institutional Animal Care and Use Committee, male Sprague-Dawley rats (Harlan Laboratories, Dublin VA) were anesthetized with urethane diluted in 0.9% sodium chloride solution and injected intraperitoneally at a dose of 1-1.5 gram per kilogram of body weight. Urethane was chosen because it is easy to use, long lasting, and does not contribute appreciable physiologic effects in the vasculature. The drug was administered in incremental doses until adequate anesthesia was achieved, this schedule of administration avoids overdosing and normal cardiovascular physiology is maintained. Animals were positioned on the surgery table and the proximal trachea was isolated. A small incision was made between tracheal cartilage rings and a polyethylene tube (PE205) was inserted into the trachea to ensure sufficient and adequate airflow throughout the experiment. The incision was then extended laterally towards the right clavicle until the jugular vein was isolated. A catheter was inserted and secured for later infusion of treatments into the venous bloodstream.

The animals were repositioned and an incision was made through the lateral aspect of the scrotum for cremaster muscle isolation. The fascia overlying the cremaster was resected and the muscle incised through the midline until its inner contents were exposed. The spermatic cord and its components were ligated and resected. The muscle was completely isolated from other tissues and spread over a glass pedestal, secured with 6-0 silk sutures and a glass cover slip was placed over it for efficient transillumination.

The animals were transferred to a compound microscope equipped for videomicroscopy. A nomenclature system was used in which arterioles are numbered according to their branch order, A1 being the first branches off the main feeding artery to the tissue, second order branches are named A2 and so on. Chosen for study were A2 (45-65 µm) and A3 (20-45µm) arterioles with clearly defined walls.

Animals were allowed to stabilize for one hour to allow the vasculature to recover from any transient changes the surgical trauma and environment temperature exposure might have induced. The muscle was maintained inside the confines of the glass cover slip on top and gauze strips secured on the sides with silk sutures. This allowed for physiologic tissue temperature control to take over. Our preliminary studies showed that tissue temperature was maintained within physiologic levels and therefore temperature-controlled chamber was not necessary. Infusion of a defined volume of resuscitation fluid (2.25 ml/100 g of body

weight) was given without hemorrhage (this method is referred to as a “topload”). The fluid was warmed to body temperature prior to injection and infused through the jugular access over a period of two minutes. This slow infusion rate was used to avoid a localized increase in blood viscosity. The videomicroscopy equipment was used to measure arteriolar diameter prior to infusion and at five-minute intervals up to thirty minutes after infusion. One to three vessels were chosen per rat depending on how clearly the vessel walls were visualized. When several vessels were measured, the changes were averaged for that rat thus, every “n” in this study represents the changes in one study subject.

The animals were randomly assigned daily to a different treatment group to receive intravenous infusion. A pilot study was originally done to compare KRF 1X, Hetastarch and PBS. These data were included in this study, hence the difference in sample sizes between treatment groups:

1. Crude keratose extract solution dialyzed with a 30 KDa NLMWCO cartridge to exclude all compounds below that molecular weight and formulated half as viscous as normal human blood (Keratose Resuscitation Fluid or “KRF” 0.5X).
2. Same as solution #1 but formulated at a viscosity similar to that of normal human blood (KRF 1X).
3. Same as solution #1 but formulated at a viscosity one hundred percent higher than that of normal human blood (KRF 2X).
4. KRF 1X after bathing the muscle with L-Nitro-Arginine (L-NA) in order to inhibit NO Synthase activity.
5. 6% Hetastarch in 0.9% Sodium Chloride solution.
6. Control group treated with the vehicle only, phosphate-buffered saline (PBS).

In order to determine the origin of the compound(s) responsible for vasodilation, a follow up experiment was conducted in which all low molecular weight keratose molecules (i.e. hydrolysis by-products) were eliminated by dialysis and a high molecular weight fraction of alpha+KAP complexes were fractionated. This was accomplished by removing the crude keratose extract immediately after 100KDa NLMWCO dialysis, titrating the solution to pH 6.0, and loading the sample onto a glass column containing anion exchange resin (Q Sepharose, General Electric Healthcare, Fairfield, CT). The resin was used according to the manufacturer's instructions and was conditioned with three volumes of 10mM tris at pH 6.0. After loading the sample, the column was rinsed with an additional three volumes of 10mM tris buffer. The flow through and rinse solutions representing the alpha fraction were collected, dialyzed at 100KDa NLMWCO, and processed to a dry powder as previously described. The sample bound to the resin representing the KAP fraction was eluted with three volumes of 100mM tris at pH 8.0 + 2M sodium chloride, dialyzed at 30KDa NLMWCO and further processed to a dry powder as previously described.

The following additional fluids were prepared and tested in the rat cremaster model through intravenous infusion as well:

- 7 Purified alpha-keratose fraction described above, formulated at a viscosity similar to that of normal human blood (Alpha)
- 8 Purified KAP fraction described above, formulated at a viscosity 2.5 times that of normal human blood (KAP)

KAP was applied both i.v. as described above, and topically. Topical application was done using a 27G hypodermic needle (BD PresicionGlide™), which was inserted between the

muscle and the glass pedestal. This allowed the fluid to be applied without lifting the glass cover and the puncture was small enough that bleeding was avoided.

Statistical Analysis

A two-way analysis of variance (ANOVA) was conducted on vessel diameter measurements and Bonferroni post-hoc comparisons were performed when appropriate to determine differences between the treatments at each time point. All graphs indicating arteriolar diameter change show increase over baseline (e.g. 1.2 indicates a 20% increase in diameter) and represent the mean with error bars indicating standard error of the mean (SEM) unless otherwise stated. Significant differences are noted in the figures.

Results

SEC analysis of the keratose samples used in these experiments showed a heterogeneous mixture of proteins. Crude keratose extract prepared by 30KDa NLMWCO dialysis had a bimodal distribution with peak apexes at approximately 60 and 200KDa, indicating the presence of monomeric and tetrameric forms of keratin (Figure 1A). Purification by IEx resulted in a notable shift in this distribution. While the 100K dialyzed sample before IEx fractionation (Figure 1B) showed multiple peaks, the alpha fraction (Figure 1C) showed a bimodal distribution but with peak apexes at 67 and 270KDa, indicating a shift to higher molecular weight compared to Figure 1A. The KAP fraction (Figure 1D) was comprised primarily of a large peak at 47KDa with a slight shoulder at 28KDa, indicating the presence of monomeric alpha keratin and KAP, respectively.

KRF 1X i.v. administration induced vasodilation that was apparent under optical microscopy (Figure 2). The effect plateaued 15 minutes following infusion and was statistically significant at all time points after 5 minutes, with a maximum average increase in diameter of 37.4% observed at 15 minutes (Figure 3). Hetastarch administration induced vasodilation that plateaued after 20 minutes with a maximum averaged effect of 12.4% increase in vessel diameter observed at 20 minutes. Equivalent volumes of PBS did not elicit significant changes in vessel diameter (Figure 3). Moreover, there were no statistically significant differences between responses elicited by Hetastarch and PBS (Figure 3). Cremaster muscles pre-treated with topical L-NA were observed for 30 minutes after application and exhibited a small decrease in diameter within the first 5 minutes; this decrease plateaued after 5 minutes and was not statistically different from levels before application. After 30 minutes KRF 1X was administered i.v. and no significant change in vessel diameter was observed (Figure 4). Altering the viscosity of KRF produced differing effects on vasodilation after i.v. infusion. KRF 0.5X did not induce statistically significant changes but KRF 2X did elicit statistically significant changes in microvascular diameter similar to KRF 1X (i.e. KRF 1X and 2X were not statistically different; $p = 0.87$) (Figure 5).

The preceding experiments using KRF demonstrated the effect of a fluid containing solute molecules composed of monomers, dimers and higher oligomers, which exist in solution as associated molecules. The experiments using the purified fluid were intended to find individual effects of these compounds. Interestingly, the purified fluid (Alpha) only induced a 2.3% increase in vessel diameter even though it was formulated at the same viscosity as the unfractionated sample and infused in the same manner (Figure 6). This observation spurred further investigation of the KAP fraction as a potential mediator of vasodilation. An additional experimental group was tested wherein a fluid at 2.5X viscosity (200 mg/ml) containing only the purified KAP fraction (KAP) in PBS was injected IV. All other experimental parameters were as described previously. Intravenous administration of the KAP fluid resulted in a rapid, transient increase in vessel diameter of 7.6% (Figure 6). The effect was substantially diminished by 20 minutes and it was noted by a change in urine

coloration that the keratose was quickly cleared through the renal system. These experiments showed that the KAP fraction induced statistically different changes from the Alpha fraction ($p < 0.0001$), but the statistically significant difference is lost at the 30 minutes time point with the observation of rapid clearance. To further confirm the KAP fraction's potential effect, the KAP solution was administered topically, directly to the cremaster muscle. This was done using a 27G hypodermic needle (BD PresicionGlide™), which was inserted between the muscle and the glass pedestal; this allowed the fluid to be applied without lifting the glass cover and the puncture was small enough that bleeding was avoided. In these experiments, enhanced vasodilation was achieved (17.75% increase in vessel size, Figure 6), this effect was statistically different compared to the same compound when infused intravenously ($F=18.03$, $p = 0.0001$) as well as to the Alpha ($F=58.59$, $p = 0.0001$).

In all cases, rats did not demonstrate any adverse reactions to the infusion of the treatments such as sudden death, seizures, blood flow alterations (i.e. emboli) or increased neutrophil adherence as noted on microvascular observations of venous flow. Animals were euthanized according to the protocol after the experiments were concluded.

Discussion

Keratins are a family of fibrous, intermediate filament forming structural proteins that self-assemble by coordination of type I (acidic) and type II (basic) monomers into obligate heterodimers [14]. These dimers self-assemble to form stable tetramers, two tetramers form an octomer [2] and four octamers complex to form a cylindrical unit length filament (ULF). End-to-end coordination of ULFs can result in the formation of 10nm diameter intermediate filaments [7]. In the samples tested in this study, monomers, dimers, and higher ordered oligomers were evident from SEC analysis. Interestingly, the KAP fraction appeared to remain complexed to these oligomers during extraction and dialysis. Only after IEX fractionation was a lower molecular weight fraction of these associated proteins able to be isolated.

The rat experiments demonstrate that KRF formulated at a viscosity similar to blood viscosity induces arteriolar vasodilation on the skeletal muscle vascular bed in a normovolemic rat. This vasodilation is mediated, at least in part, through the NO pathway as demonstrated by the fact that after inhibiting NO synthase with LNA, KRF 1X did not induce any change in arteriolar size (Figure 4). Low viscosity (i.e. 0.5X) KRF induced less vasodilation compared to fluid with increased viscosity (achieved by increasing concentration), which resulted in significantly greater vasodilation (Figure 5). This relationship suggests that one or both of the following mechanisms may mediate vasodilation:

1. vascular shear stress at the vessel wall, or
2. pharmacologic stimulation of endothelial NO production

In order to investigate the source of the keratin responsible for this activity, chromatographic separation was performed so that the alpha and KAP fractions could be studied separately. The alpha fraction is made up of large molecules that contribute viscosity to the solution. The KAP materials bound to the anionic exchange column are small molecules so they would be expected to contribute little to no viscosity to the fluid. Data in Figure 3 demonstrate that persistent vasodilation is achieved with the fluid containing alpha plus KAP, but eliminating KAP from this molecular complex significantly reduces the vasoactive effect, as shown in Figure 6. Moreover, when a KAP only fluid is infused, a transient vasodilation effect is noted with rapid clearance of these smaller molecules. This suggests a pharmacologic-mediated action from the KAP fraction. To further confirm the KAP fraction's potential vasoactivity, the KAP solution was administered topically, directly to the

cremaster muscle. In these experiments, a mean increase of 17.75% in vessel diameter was observed (Figure 6). However, this effect was muted when compared to the KRF 1X fluid but was potent enough to demonstrate a statistically significant difference compared with the same compound infused intravenously ($F=18.03$, $p = 0.0001$). This suggests that the KAPs' efficacy is dependent on it being able to form a molecular complex with the larger alpha keratose molecules.

Whether the vasoactive effect is in fact achieved by one of the mechanisms mentioned above or a summative effect of both will require further investigation. However, the fact that the Alpha compound failed to elicit vasodilation when formulated at the same viscosity and infused i.v. in the same manner, points toward a receptor-mediated mechanism rather than a viscosity effect. Moreover, the fact that the effect was more pronounced when KAP was complexed with the larger Alpha molecules suggests that the Alpha is either serving to increase the circulating half-life of the KAP compound, or the formation of the molecular complex itself renders the KAP molecules more active, perhaps through some specific folding interaction.

Changes in the concentration of alpha-keratose allow for adjustment of solution viscosity and oncotic characteristics in the KRF. Data from fluids ranging in viscosity from 0.5X (1.7 cP) to 2X (9.7 cP) demonstrated more of a threshold effect than a dose response (Figure 5). This suggests that viscosity plays a smaller role relative to the pharmacologic effect of the KAP fraction. However, the KAP fraction appeared to have been cleared quickly, thereby diminishing the persistence of vasoactivity. Interestingly, topical application appeared to induce stronger vasodilation than i.v. injection, although it was weaker than infusion of the combined alpha+KAP, KRF 1X. These observations again suggest that the complex of alpha and KAP is important for vasoactivity. Binding to a keratin oligomer would appreciably increase the effective molecular weight of the alpha+KAP complex, thereby increasing its circulation half-life. Preserving structural integrity of these complexes may also be important for presenting active sites that are needed for pharmacologic efficacy. Interestingly, other keratin preparations have been shown to have hemostatic characteristics [1], whereas keratoses seem to be completely blood and cardiovascular compatible. This further suggests that tertiary structure of the alpha+KAP keratose complex is an important aspect of vasoactivity.

While these observations for keratins are novel, vasoactive peptides are not a new discovery. Angiotensin is a strong vasoconstrictor, kinins are vasodilators, and vasoactive intestinal peptide has several roles, including vasodilation. All of these compounds have been reviewed elsewhere and have complex mechanisms [4, 8, 11]. Interestingly, antihypertensive effects have been associated with several materials including peptides derived from dietary sources [22], as well as another biomaterial, chitin [9]. In a similar investigation to the present work, Miguel et al. showed vasodilator activity associated with several peptides from egg whites [12]. The peptide sequences IVF, RADHPFL, YAEERYPIL, YPI, and RADHP all induced vasodilation in intact aortic rings, up to a maximum of >50%. However, none of these sequences are present in the human trichocytic keratin proteome with the exception of YPI, which is found in one KAP molecule, KRTAP7-1 [17], a KAP not found in our human hair keratin extracts [5].

Conclusions

This work represents the first observations of vasodilation activity associated with human trichocytic keratins. KRF administered intravenously caused rapid, sustained vasodilation when the form of the keratin was a supramolecular complex comprised of both alpha and KAP keratose derivatives. The vasoactivity appeared to be more pharmacologic than shear

mediated, and could be isolated to a purified KAP fraction consisting of a relatively low molecular weight fraction. Both i.v. and topical application of this material induced vasodilation. Experiments using L-NA implicated the NO pathway, although a definitive mechanism deserves further study.

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Abbreviations

KRF	Keratin Resuscitation Fluid
KAP	Keratin-Associated Protein
NO	Nitric Oxide
i.v. or IV	Intra-Venous
DI	Deionized
NLMWCO	Nominal Low Molecular Weight Cutoff
SEC	Size Exclusion Chromatography
HEPES	Hydroxyethyl-Piperazine Ethanesulfonic Acid
L-NA	L-Nitro-Arginine
PBS	Phosphate Buffered Saline
ANOVA	Analysis of Variance
IEx	Ion Exchange
KDa	KiloDalton
ULF	Unit Length Filament
cP	Centipoise
Abs	Absorbance

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Figure 1. Size exclusion chromatography of keratose samples

The sample used in test groups 1-4 (A) indicates the presence of monomeric and tetrameric forms of alpha keratin. Before purification, many peaks are present in the 100K dialyzed crude extract (B), indicating multiple protein species. After fractionation by ion exchange chromatography the higher molecular weight alpha (C) and lower molecular weight KAP (D) fractions are evident (K = KDa, Abs = Absorbance, nm = nanometers).

Figure 2. Video stills of the same vessel before and after KRF administration

The image on the left shows a blood vessel before KRF administration while the one on the right shows the same vessel after i.v. injection of KRF 1X. Magnification is the same in both images.

Change in Arteriolar Diameter with Infusion of IV Resuscitation Fluids

Figure 3. Change in arteriolar diameter with i.v. administration of Fluid
 KRF 1X induced a maximum vasodilation of 37%. Hetastarch induced vasodilation of 12%; the difference in relative response between these compounds was statistically significant. There was no statistically significant difference between the effects of hetastarch and PBS. Data is presented as a percentage of baseline (statistical difference noted is between treatment groups).

Effect on Arteriolar Diameter of IV KRF with and without L-Nitro-Arginine

Figure 4. Effect on arteriolar diameter with and without L-nitro-arginine

The vasodilator effect of KRF IX was blocked with L-Nitro-Arginine. This demonstrates that NO production plays a role in the vasodilator effects of KRF administered intravenously (statistical difference noted is between treatment groups).

Change in Arteriolar Diameter with IV Infusion of Keratose fluids of Different Viscosities

Figure 5. Average arteriolar diameter increase with KRF of different viscosities

Low viscosity KRF induced less vasodilator effect while increasing viscosity resulted in an increase in vasodilation (0.5X [n=5], 1X [n=11], 2X [n=5]) when administered intravenously (statistical difference noted is between groups, no difference was noted between KRF 1X and KRF 2X).

Change in Arteriolar Diameter with IV Infusion of Purified Alpha KRF vs Purified KAP Fraction Applied IV and Topically

Figure 6. Average arteriolar diameter increase with infusion of Alpha and KAP containing fluids
 The KAP fraction showed a modest, transient increase when applied intravenously which was statistically higher than the Alpha fraction. Alpha compound did not induce a significant increase in vessel diameter. The KAP fraction's relative effects were increased when applied topically, which is demonstrated by a statistical difference when compared to the KAPs IV.