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Review article

Effects of dietary sports supplements on metabolite accumulation, vasodilation and cellular swelling in relation to muscle hypertrophy: A focus on "secondary" physiological determinants

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ABSTRACT

Increased blood flow via vasodilation, metabolite production, and venous pooling contribute to the hyperemia and cellular swelling experienced during resistance training. It has been suggested that these effects play a role in hypertrophic adaptations. Over the past 2 decades, sport supplement products have been marketed to promote exercise hyperemia and intracellular fluid storage, thereby enhancing hypertrophy via acute swelling of myocytes. The three main classes of supplements hypothesized to promote exercise-induced hyperemia include vasodilators, such as nitric oxide precursor supplements; anaerobic energy system ergogenic aids that increase metabolite production, such as β-alanine and creatine; and organic osmolytes, such as creatine and betaine. Previous studies indicated that these dietary supplements are able to improve muscle performance and thus enhance muscle hypertrophy; however, recent evidences also point to these three classes of supplements affecting "secondary" physiological determinants of muscle mass accretion such as vasodilation, metabolite accumulation, and muscle cellular swelling. Although we recognize that the literature is relatively scarce regarding these topics, a better comprehension and discussion of these determinants can lead to increased knowledge and might guide further research regarding the proposed mechanisms of action of the identified compounds. In this case, increased knowledge may contribute to the development of improved efficacy, new products, or direct new research to specifically investigate those secondary effects. The aim of this review was to bring into focus new perspectives associated with secondary physiological effects induced by supplementation and to determine their relevance.

Introduction

Skeletal muscle remodeling is an adaptive process dependent on mechanical and chemical factors affecting muscle protein accretion [1]. The optimal relative intensity suggested by the American College of Sports Medicine to induce a robust muscle hypertrophy effect is ~75% of the 1 repetition maximum (RM) [2]. However, several recent studies performed with and without blood flow restriction (BFR) resistance training have demonstrated that muscle hypertrophy can occur while using loads as low as 20% to 40% 1 RM. In these instances, the main mechanisms driving the muscle hypertrophy process are hypothesized to be increases in

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blood flow and metabolite accumulation via the contracting muscle [3]. During and after a maximal low-load and high-repetition resistance training bout leading to a momentary concentric failure, it is expected that intramuscular adenosine triphosphate (ATP) levels fall ~30% to 40%, with creatine phosphate (CP) stores being significantly reduced (and eventually depleted), whereas plasma byproducts of muscle contraction such as adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, nitric oxide (NO), and lactate will be increased. These metabolites increase blood flow to the contracting muscle (acting either as direct or indirect vasodilators); however, an increase in blood flow alone likely does not seem to enhance muscle hypertrophy [4]. It is the combination of decreased ATP and CP stores (and inorganic phosphate (Pi), ADP, and adenosine monophosphate accumulation), a high glycolytic flux (increased hydrogen ion production, leading to metabolite accumulation), moderate hypoxia (via BFR and increased muscle contraction), and venous pooling leading to cellular swelling that may enhance hypertrophic adaptations.

Some nutritional supplements are consumed with the intention of enhancing muscle hyperemia, and thus optimizing the hypertrophic response, for goals pertaining to athletic performance and aesthetic appearance. Although different compositions and propriety blends are available on the market, common ingredients such as L-citrulline (a non-essential amino acid) or L-arginine, have been popularly marketed with the premise that they act as vasodilators [5,6] that will enhance hyperemia. Another common component of nutritional supplements marketed to enhance vasodilation is dietary nitrate, commonly consumed in the form of nitrate-rich beetroot juice [7]. Classical performance-enhancing supplements such as creatine monohydrate and β-alanine are also included within many propriety blends [8,9]. Although these compounds show important muscle-buffering properties, the same substances also may enhance anaerobic energy production and total work [9,10]. This, in turn, would increase metabolite accumulation and the vasodilatory response, thus possibly amplifying muscle hypertrophy via secondary mechanisms. Finally, the osmotic properties of supplements such as betaine and creatine may contribute to increased water content within a cell, thus increasing cellular swelling [9,11,12]. The objective of this review is to summarize current information regarding the use of ergogenic aids as potential strategies to acutely increase cellular swelling, vasodilation, and metabolite accumulation in response to resistance exercise and to discuss underlying mechanisms of action by which these supplements may potentially enhance muscle hypertrophy. Although we recognize that the literature is relatively scarce regarding the effects of nutritional supplements on muscle hyperemia and the resulting physiological "secondary" determinants of muscle hypertrophy, these topics are herein described because we believe that a better comprehension and discussion of these effects and mechanisms of action can lead, in the future, to more mechanistic research related to the proposed secondary effects. This, in turn, may not only lead to a better association of those supplements with specific resistance-training regimens, but also aid in the development of new products specifically designed to enhance those secondary effects, if relevant.

The role of cellular swelling in muscle hypertrophy

Several years ago, muscle overload was believed to be the major force driving muscle hypertrophy. Also, previously, an association between the types of muscle action (mainly eccentric exercises) and muscle damage were described as essential to build muscle [13]. Currently, it is believed that a muscle may robustly hypertrophy either in the absence of heavy external loads or eccentric actions with minimal muscle damage. The main physiological mechanism responsible for such achievements is the so-called "metabolic stress," a secondary event from low-intensity muscle contractions (to failure), which, if repeatedly performed, can lead to muscle growth [14]. Regarding metabolic stress, it was once thought that the association between metabolic stress and hypertrophy was non-causal in that greater mechanical work resulted in higher levels of metabolites, and greater mechanical work was responsible for hypertrophy. Dankel et al. [15] hypothesized that the metabolites produced during resistance exercise are not directly anabolic, but rather permit muscle protein synthesis in a wider range of muscle fibers by increasing motor unit activation and augmenting the mechanotransduction cascade. Recently, however, new research suggests that metabolites may work independently of, or synergistically with, muscle contraction to promote hypertrophy. For example, Ohno et al. [16] demonstrated that extracellular lactate increased C2C12 cross-sectional area in a dose-dependent manner by activating the mitogen-activated protein kinase kinase 1/2 (MAP2K1) pathway.

There is a plethora of different biological factors accumulating in a contracting skeletal muscle speculated to lead to muscle hypertrophy, with cellular swelling being one of them. In response to vigorous resistance exercise, cellular swelling is characterized by the summation of a moderate venous pooling effect (owing to venous compression induced by muscle contraction) and increased vasodilation of arteries, arterioles, and capillaries (owing to decreased muscle tissue oxygenation or increased vasodilatory metabolites, including lactate) [17]. This results in a fluid shift from the capillaries into the interstitial space, in part because of an increase in the vascular and extracellular pressure gradients [14]. As described by Schoenfeld and Contreras [18], this enhanced reperfusion causes muscle tissue to become engorged with fluid in a phenomenon commonly referred to by sports scientists as *exercise-induced hyperemia* and by bodybuilders as *the pump*. During submaximal resistance exercise, the increase in muscular volume appears to be the result of increases in extracellular water, whereas during maximal resistance exercise, fatigue increases in muscle volume are predominantly the result of increases in intracellular water [19]. The observed shift in fluid from the vascular-interstitial space during exercise exceeds the rates predicted by changes in hydrostatic capillary pressure alone [20], which has led to the suggestion that increases in muscle water volume during exercise above transcapillary flow are the result of intracellular osmoles [19,21]. Although there are a variety of active intracellular osmolytes, creatine, lactate, inorganic phosphate, and sodium and chloride increase the most during exercise and contribute to exercise-induced cellular swelling, with lactate hypothesized to have one of the largest effects [22]. However, a recent study conducted in resting single amphibian muscle fibers places doubt on this hypothesis. In this study, intracellular lactate and hydrogen ions (H^+) were matched to concentrations observed during muscle fatigue and did not produce an increase in intracellular water [23]. The authors proposed that increases in H^+ during glycolysis offsets any lactate-induced increases in cellular water influx. On the other hand, Kemp [24] argued that during glycolysis, phosphocreatine catabolism via creatine kinase to maintain ATP balance (sometimes termed the *Lohmann reaction*) opposes acidification [25], increases inorganic phosphate, which tends to reduce the negative charge of membrane to impermeant anions [26], produces the osmolyte creatine, and thereby results in a positive glycolytic cellular swelling effect. Although the magnitude to which individual metabolites contribute to cellular swelling is beyond the scope of this review, there is considerable evidence that metabolites potentiate muscle hyperemia and the cellular swelling effect [14,19,21].

Probably the best-known example to illustrate the potential influence of cellular swelling on muscle hypertrophy (although not

completely isolated from other variables), is the Kaatsu walk exercise. Kaatsu, also known as BFR involves applying tourniquets/cuffs proximally on the limb (generally the thighs or upper arms) and inflated to partially or fully restrict the outflow of blood from the muscle during exercise. It is widely known, for example, that walking activities rarely produce muscle hypertrophy responses in the contracting leg muscles. However, walking with BFR (Kaatsu walk), even at a low speed, is capable of producing a significant amount of muscle hypertrophy [27]. The main reasons responsible for such response seem to be the increased muscle fluid volume caused by the use of BFR, paralleled by moderate increases in metabolite accumulation [27]. In support of the acute increase in muscle hyperemia influencing muscle protein synthesis, it has been demonstrated that low-load (20% 1 RM) resistance exercise (load- and volume-matched with a BFR paradigm) does not produce the same increases in post-exercise muscle protein synthesis observed by the BFR paradigm [4]. The superiority of the BFR method, even when total muscle blood flow was pharmacologically matched [4], seems to be related to the greater immediate blood flow response during reperfusion (the first 10 min after the removal of the cuffs) or a possible fluid shift/cell swelling to the muscles in favor of the BFR method [4].

As discussed previously, low-load BFR resistance training results in increased rates of muscle protein synthesis when compared with load- and volume-matched resistance exercise. Such effects may be partly due to increased fluid shift to the muscles. In this regard, Loenneke et al. [28] demonstrated that by just applying a cuff to the proximal region of the lower limb and inflating the cuff (similarly with that employed in rehabilitative studies showing antiatrophy effects), acute increases in muscle thickness were observed. Although it is uncertain whether intracellular water was changed with such procedures, plasma volume was substantially decreased, suggesting that there was a fluid volume shift from plasma to the muscle tissues [28]. In addition, removal of the cuff still maintained increased muscle thickness for 3 min, suggesting a relationship between acute increases in muscle thickness and cell swelling mechanisms [28]. During exercise conditions, acute increases in the muscle thickness were demonstrated to be superior for the BFR with equal load but less volume when compared with a low-load resistance-training paradigm, suggesting a similar mechanism of increased cell swelling during exercise conditions [29]. It is still unknown, however, exactly how acute changes in cell swelling and muscle fluid shift caused by resistance exercise influences muscle anabolism. The following summarizes some of the possible mechanisms.

As previously reviewed by Schoenfeld and Contreras [18], there is some evidence to support a mechanistic link between cellular swelling and skeletal muscle hypertrophy. This acute myocyte swelling is a physiological reinforcement in the contracting muscle, which positively influences the muscle hypertrophy response [3,29], likely as a summation of both direct and indirect anabolic actions. Cellular swelling has been shown to stimulate protein synthesis in mammary [30] and hepatic cells [31]. In hepatocytes, the rates of protein synthesis and degradation are controlled by cell swelling mechanisms [32]. In this regard, it has been shown that induction of cell shrinkage, caused by extracellular hypertonicity, induces a robust decrease in protein synthesis rates when compared with an isotonic condition [31] and a similar phenomenon has been shown to occur in skeletal muscle in vitro [33]. Häussinger [32] hypothesized that cellular swelling is sensed by integrins and transduced downstream signal protein synthesis, gene transcription, and inhibit proteolysis via MAPK. Cellular swelling may also augment protein stimulus because cell hyperhydration has been shown to enhance amino acid in skeletal muscle uptake via phosphatidylinositol 3-kinase activation [34].

Studying protein metabolism via cell swelling mechanisms may be important not only for diseased cellular physiology, but also to understand the effects of resistance exercise at the cellular level. Although numerous cell types have been described to be affected by cell swelling mechanisms [32,35], in vivo studies, especially those performed in humans, are scarce in the literature. In a recent study performed by Mora–Rodríguez et al. [36], the effect of a prolonged dehydrating exercise session (150 min at 33°C ± 1°C, 25% ± 2% humidity) on muscle water content was evaluated. For this purpose, nine endurance-trained cyclists had their vastus lateralis muscles biopsied before, immediately after, and 1 and 3 h after a dehydrating exercise. Right after exercise, muscle water content remained at pre-exercise values. However, after 1 h of supine rest, muscle water content was reduced by 13% and remained diminished after another 3 h, compared with pre-exercise values [36]. Such results demonstrate that muscle water content can be affected by exercise conditions, probably in an attempt to maintain plasma volume [36]. It is not known from this study whether muscle dehydration was correlated with catabolism. However, in another study performed with humans, changes in extracellular osmolality were induced by administration of either a vasopressin analog plus liberal water drinking (to induce hypo-osmolality), or infusion of a hypertonic saline solution and restriction from drinking (to induce extracellular hyperosmolality) [37]. Although not directly measured, the authors suggested that the extracellular hypo-osmolality induced a modest state of cell swelling, whereas the hypertonicity resulted in cell shrinkage [37]. As a result, protein breakdown was diminished during hypo-osmolality/hyperhydration compared with iso-osmolality [37]. Although protein metabolism was only measured at the whole-body level (reflecting both muscle and non-muscle tissues), the results suggest that even in humans, cell swelling is a mechanism that affects muscle protein metabolism [37].

Muscle hypertrophy, cell swelling, and related-mechanisms of action

During resistance exercise, arterial blood flow to the active musculature increases, whereas forceful contraction causes venous compression. As a result, fluid pools in the active musculature, resulting in a transient cellular swelling effect that increases the volume of myocytes [18]. It has been speculated that the increased muscle water retention will increase strain on the sarcolemma, thereby stimulating protein synthesis through mechanotransducing signaling [29]. Given that homeostatic regulation of cell volume is imperative to proper function in a variety of cells [38], it also has been suggested that transient, exercise-induced cellular swelling may present a threat to the myocyte's structural integrity, thereby promoting an adaptive expansion of the cell by means of hypertrophy. In response to cellular swelling, anabolic processes are upregulated and catabolic processes are downregulated through mechanisms mediated by integrin-associated volume osmosensors present within the myocyte [18,34]. In this regard, the evidence is provided by the activity of aquaporin-4 (AQP4), a water transport channel that is particularly abundant in type 2 muscle fibers [39]. Because muscle atrophy has been observed the activity of aquaporin-4–knockout mice [40], a role for muscle water transport and muscle mass control has been established. However, in the hypertrophy process, such molecular relationships are still lacking. Overall, it has been proposed that increased cell swelling may partially explain augmented hypertrophic adaptations in response to BFR exercise [3]. If true, enhanced cell swelling may provide a mechanism by which dietary supplements that increase exercise hyperemia, enhance anaerobic energy systems, or exert osmotic effects, may increase hypertrophic adaptations to resistance training.

Increasing cellular swelling through ergogenic supplements

Presently, there are several dietary/sports supplements that are widely consumed in hopes of accelerating or optimizing skeletal muscle hypertrophy in an additive/synergistic effect when combined with high-intensity muscle contractions. In this regard, it has been speculated that three main classes of substances are capable to increase the cellular swelling effect through distinct mechanisms of action:

1 Supplements with vasodilator properties.
2 Supplements that enhance anaerobic energy systems.
3 Supplements with osmotic properties.

Some supplements seem to fulfill two or more requirements and may act via multiple mechanisms of action in the cellular swelling response (Fig. 1).

Supplements with vasodilator properties: NO precursor supplements

NO is a signaling molecule with multiple functions throughout the body, most notably recognized as a potent stimulator of vasodilation. As reviewed by Bailey et al. [41], NO-mediated effects on exercise efficiency, mitochondrial respiration, calcium handling, vasodilation, glucose uptake, and muscle fatigue have prompted great interest in the use of NO precursor supplements as a means of enhancing acute exercise performance. Although the acute effects of NO precursors on exercise performance have been studied extensively, there is a lack of data investigating the effects of NO on skeletal muscle hypertrophy, despite multiple plausible mechanisms for potential roles. Previous research has demonstrated that overload-induced alterations in skeletal muscle size and fiber type are blunted in rodents with blocked production of endogenous NO [42], and that NO release exerts direct effects promoting the activation of satellite cells [43], which is critical for the initiation of muscle hypertrophy. As a result, administration of a nitric oxide donor (isosorbide dinitrate) in addition to voluntary exercise increased exercise-induced hypertrophy of the quadriceps in mice compared with exercise or isosorbide dinitrate alone [44]. In the context of resistance training, NO also may indirectly influence hypertrophy outcomes by delaying fatigue and allowing a greater volume of total work to be completed during fatiguing exercise, thereby conferring a more robust stimulus for muscle growth. Finally, it previously has been suggested that cell swelling and exercise hyperemia may influence hypertrophy by applying mechanical pressure against the myocyte membrane and facilitating the delivery of anabolic growth factors and hormones to satellite cells, respectively [13]. As such, a NO-mediated increase in blood flow during exercise may potentially augment these mechanisms contributing to skeletal muscle hypertrophy.

NOS-dependent precursors

NO may be formed via nitric oxide synthase (NOS)-dependent or NOS-independent pathways. The NOS-dependent pathway is considered the classical pathway of NO production, in which the precursor L-arginine is converted to NO via an aerobic process dependent on tissue-specific NOS isozymes and a number of requisite cofactors [41]. The role of L-arginine in this pathway has prompted interest in arginine supplementation to enhance blood flow and exercise performance. Intravenous infusions of L-arginine have been shown to increase blood flow [6], whereas oral arginine supplementation generally appears to be ineffective [6,45,46], likely because of extensive pre-systemic degradation. Arginine supplementation has failed to consistently improve exercise outcomes, particularly in healthy individuals, as reviewed by Bescoet al. [5]. In contrast to L-arginine, orally ingested L-citrulline is not subject to extensive pre-systemic degradation and therefore increases plasma arginine levels more efficiently than oral L-arginine supplementation [47]. Recent research using aerobic exercise modalities has documented improvements in time to

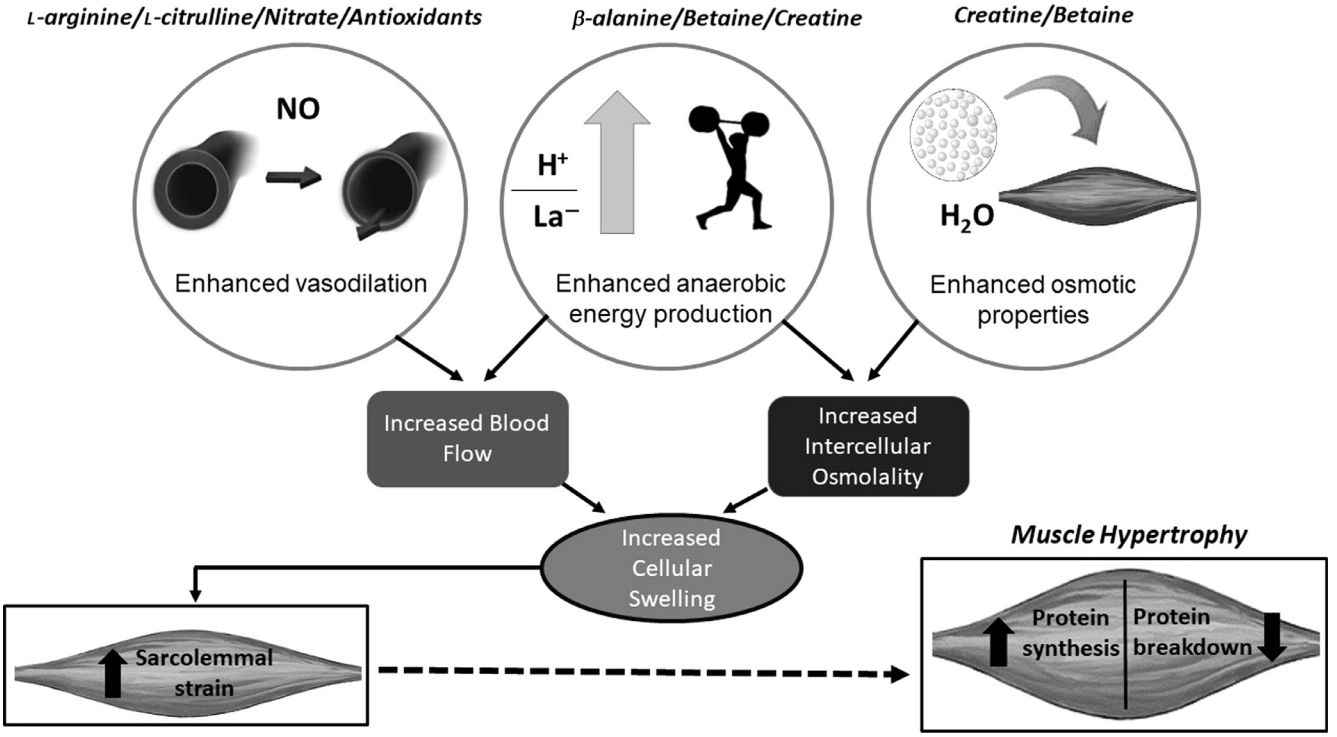

Fig. 1. Three main classes of substances capable to increase cellular swelling through distinct mechanisms of action. H^+, hydrogen ion; H_2O, water; La^-, lactate; NO, nitric oxide.

exhaustion after L-citrulline supplementation, although mixed findings have been reported [48]. Bailey et al. [49] recently conducted a study directly comparing equivalent doses of oral arginine with citrulline in healthy humans, with results indicating that citrulline had more favorable effects on oxygen kinetics, blood pressure, time to exhaustion, and total work performed during a high-intensity cycling test. Emerging research has investigated the effects of citrulline malate on acute resistance exercise performance, with results showing improvements in repetitions completed and total work performed during multiple sets taken to concentric failure [50–52]. Despite these promising results, it is unclear if citrulline malate is modifying performance through a NO-mediated mechanism, and it is not known if these acute increases in work output translate to greater hypertrophy over time. Considering that muscle hypertrophy has only been demonstrated with the use of NOS-dependent precursors in aging mice with refractory satellite cells [44], there is no human evidence that NOS-dependent precursors promote muscle hypertrophy in young, healthy muscle.

NOS-independent precursors

In the NOS-independent pathway, nitrate ($NO3^-$) is reduced to nitrite ($NO2^-$), which is further reduced to NO [41]. This pathway is particularly promising with respect to high-intensity exercise because it operates independently of NOS enzymes, functions anaerobically, and is stimulated by acidosis and hypoxia. Dietary nitrate supplementation via beetroot juice/extract or inorganic nitrate has become increasingly popular in the past decade, with studies documenting improvements in mitochondrial efficiency[53-55], exercise tolerance [53,56,57], blood flow [58], and contractile function of muscle [59,60]. These mechanisms have translated to improved time to exhaustion [53,57] and time trial performance [56,61,62] in tests using aerobic exercise modalities, such as running, cycling, and rowing. In addition, a growing body of preliminary research has suggested that dietary nitrate supplementation enhances high-intensity sprint performance [7,63–65]. One recent study [66] investigated the effects of beetroot juice supplementation on a resistance exercise test consisting of three sets of bench presses taken to concentric failure, with a load equivalent to 60% of the 1 RM. Participants completed significantly more repetitions and lifted more total weight in the beetroot condition than in the placebo group. In this study, no between-treatment differences were seen in blood lactate or perceived exertion. Thus, the acute effects of dietary nitrate supplementation seem to positively affect exercise performance in high-intensity anaerobic, aerobic [53,57], and resistance training modalities [66]. The possible mechanisms of action responsible for those improvements are increased blood flow [58], increased mitochondrial efficiency, oxygen utilization, and enhanced muscle contractile properties [41,54,55]. Although alterations in anaerobic metabolism are thought to contribute to improvements in resistance training performance, changes in blood lactate concentrations were not observed in the supra-cited study [66]. On the other hand, the ingestion of dietary nitrates has been shown to increase muscle blood flow and local oxygen consumption via local vasodilation during one set of moderate- and high-intensity handgrip exercise [67]. Future studies are necessary to investigate whether increases in resistance training volume via dietary nitrate ingestion translate to enhanced hypertrophy and how increases in blood flow during resistance exercise affect muscle hyperemia and cellular volume.

Combination with antioxidants

The use of antioxidants in combination with leucine supplementation has been shown to accelerate the regain of muscle mass after immobilization owing to increases in muscle protein synthesis [68]. Although the mechanisms are not completely known, the use of antioxidants may increase the half-life of several signaling molecules in biological tissues, including NO, via free radical scavenging mechanisms [1]. Presumably, if NO half-life is increased, antioxidants would reinforce its biological effects. In favor of these assumptions, antioxidants have been shown to exert vasodilatory actions independently [69,70] and may enhance the bioavailability of NO by preventing degradation of NO by free radicals [71]. This may help to explain why the combination of nitrate and polyphenols in beetroot juice appears to have such consistent positive outcomes in exercise research to date and why beetroot juice confers more favorable effects than a nitrate-matched dose of sodium nitrate [72]. The combination of polyphenols and nitrate may also explain why pomegranate extract, which has comparatively lower nitrate content than beetroot, was previously shown to favorably affect blood flow, vessel diameter, and running time to exhaustion [73]. Pomegranate extract also was shown to have modest positive effects on sprint performance and repetitions to fatigue in addition to blood flow and vessel diameter, although the effects on a single set of resistance exercise taken to failure were not statistically significant [74]. This potentially synergistic effect may also apply to the NOS-dependent precursors of NO synthesis, as one study has indicated that a combination of L-citrulline and the antioxidant glutathione raises plasma levels of L-arginine and NO metabolites to a greater extent than either ingredient alone [75]. The link between increased vasodilation via dietary supplements and muscle hypertrophy is likely not the result of increased nutrient delivery or tissue oxygenation/metabolite removal, but instead owing to an increased potential for cellular swelling response. As previously discussed, there are several possibilities whereby physical (use of cuffs) or pharmacologic (nitroprusside infusion—an NO donor) aids may modify fluid shift or arterial blood flow to the muscle, potentiating cellular swelling [4,14,29]. However, although many supplement manufacturers advocate increases in “muscle pump” (exercise-induced hyperemia) as a result of consuming products designed to increase muscle vasodilation, they have not been directly evaluated under resistance training conditions. To our knowledge, studies evaluating acute increases in vasodilation and cell swelling have never been simultaneously assessed. Considering that acute increases in muscle blood flow leading to acute increases in muscle thickness are correlated with cell swelling, studies designed to assess such variables would be interesting to unmask the secondary effects of such supplements.

In summary, there are mechanistic data supporting a direct effect of NO on skeletal muscle hypertrophy via satellite cell activation and the potential for NO precursor supplements to influence hypertrophy outcomes by enhancing exercise hyperemia and the total volume of work during exercise. Research has indicated that citrulline malate is effective in enhancing repetitions completed during multiset resistance exercise taken to concentric failure, but it is currently unclear if the mechanism underlying this effect relates directly to NO synthesis. Various sources of dietary nitrate have consistently been shown to enhance blood flow and aerobic exercise endurance, but there are limited data available to conclusively determine if this effect translates to high-load resistance training. Despite research documenting acute improvements in blood flow and resistance to fatigue, longitudinal, placebo-controlled training studies in humans are needed to determine if NO precursor supplements, with or without co-ingestion of antioxidants, facilitate hypertrophic responses to resistance training.

Supplements that enhance anaerobic energy production: βeta-alanine and creatine

β-alanine is a non-proteinogenic amino acid, and its synthesis occurs within the liver from the degradation of cytosine, thymine, and uracil [76]. After the breakdown of pyrimidines,

decarboxylation by gut microbes of L-aspartate, and the transamination with 3-oxopropanate by L-aspartate, it is transported to muscle cells where it is taken up through the sarcolemma via a Na^+ and Cl^- dependent process [77]. When β-alanine is combined with histidine, the dipeptide carnosine is synthesized through an ATP-dependent reaction in skeletal muscle cells [78]. Daily doses of 4.8 to 6.4 g of β-alanine can increase human muscle carnosine content by 60% in 4 wk and 80% in 10 wk [79]. Interestingly, equimolar carnosine intake does not elevate muscle carnosine more than β-alanine alone [80]. Kendrick et al. [81] compared muscle biopsies of β-alanine (6.4 g/d) and placebo-supplemented groups during 4 wk of isokinetic training (10 × 10 maximal repetitions, 90-degree extension and flexion contractions at 180 degrees/s) and reported that β-alanine availability was the main factor regulating muscle carnosine synthesis.

The ergogenic effects of β-alanine appear to be the result of reduced local fatigue during sustained muscle contractions [76]. In this regard, Severin et al. [82] were the first researchers to demonstrate β-alanine's functions as an intracellular pH buffer in both frog and in human skeletal muscle. A meta-analysis conducted by Hobsonet et al. [9] demonstrated that β-alanine increased the intramuscular buffering of H^+ mainly during high-intensity exercise lasting 1 to 4 min. At present, it is still uncertain if β-alanine supplementation enhances the cellular swelling effect. The basic premise behind β-alanine supplementation is to increase the muscle-buffering capacity, thus increasing the glycolytic energy flux during maximal/supra-maximal high- intensity exercises. With an increasing energy output capacity, a large amount of muscle metabolites can potentially be produced and will theoretically increase the cellular swelling effect. In a recent study, Bellinger and Minahan [83] demonstrated that β-alanine increased the maximal power and time to exhaustion during time trial 4000-m cycling, and these increases were associated with greater blood lactate, blood pH, and blood bicarbonate concentrations compared with placebo. However, β-alanine only marginally affected muscle lactate production. In another study, β-alanine supplementation increased muscle carnosine content, but the muscle-buffering capacity was not affected and the muscle lactate production was, in fact, decreased by 23% during intense cycling exercise [84]. At present, there is no conclusive evidence to support the role of β-alanine in enhancing muscle lactate production (or other muscle metabolites) during high-intensity contractions, and a direct effect of β-alanine on cellular swelling is currently lacking.

The effects of chronic β-alanine supplementation in combination with resistance exercise on muscle hypertrophy requires further study. Some researchers have reported increases in lean mass after chronic supplementation [85], whereas others have reported no differences between β-alanine and placebo [86]. Training status may explain this discrepancy in results; the participants in a study by Kern and Robinson [85] were trained NCAA Division I athletes, whereas those in Kendrick et al. [86] and Outlaw et al. [87] were untrained. From a mechanistic perspective, trained individuals can sustain more prolonged high-intensity muscular contractions, resulting in the generation of more metabolic by-products, thereby possibly resulting in greater cellular swelling and stimuli for hypertrophy. Independently, considering that muscle adaptations are easiest to occur in the untrained muscle, β-alanine supplementation may, in fact, potentiate muscle hypertrophy, as observed in the supra-cited study employing trained individuals. Painelli et al. [88] reported greater improvements in anaerobic Wingate performance in trained versus untrained cyclists. However, lactate concentrations were not reported and studies comparing the ergogenic and metabolic effects of β-alanine supplementation in resistance training protocols need to be conducted to substantiate this hypothesis.

Carnosine analogs have been developed with the premise that carnosine can be conjugated with other molecules thus increasing its stability and half-life (endogenous carnosine is rapidly inactivated by carnosinases), thus conferring completely new properties to the carnosine molecule. In this regard, conjugation of carnosine with several types of organic molecules has the main purpose of reducing the carnosinase action and promoting synergism with the peptide properties as well. Different modifications of the carnosine structure have been performed, generating a number of multifunctional druglike molecules such as carnosine nitro-oxy derivatives or carnosine conjugated with metals, such as copper (carnosine [Cu^+]) [89]. According to preliminary assays, carnosine nitro-oxy derivatives promotes a relaxation effect of endothelium-denuded rat-aorta strips precontracted with phenylephrine treatment [89]. The same effect was obtained with the dietary dipeptide carnosine (β-alanine−L-histidine), an effect mediated via increased cyclic guanosine monophosphate mechanism [90]. Interestingly, in the same isolated arterial preparations and range of concentration, L-histidine treatment was ineffective in inducing vasodilation, whereas β-alanine induced a vasoconstriction response. Thus, the balance between β-alanine and β-alanine dipeptide concentrations may play a role in the control of vascular tone. To the best of our knowledge, no in vivo study has evaluated the role of any supra-cited molecule in the cellular swelling effect. However, in an in vitro study, treatment of C2C12 muscle fibers with β-alanine (5 nM) increased intracellular osmolarity and water content but not myosin heavy chain II content [91]. Given that intracellular acidification reduces cell volume [92], by buffering intracellular H^+ and attenuating reductions in cellular pH, β-alanine may further the cellular swelling effect of resistance exercise. Whether increases in intracellular volume combined with resistance exercise are responsible for some of the hypertrophic effects seen with β-alanine supplementation require further testing.

Creatine

Creatine is a heterocyclic amine able to modulate various biological processes in skeletal muscle at the cellular and structural level. At the metabolic level, the phosphagen-derived energy is provided during high-intensity muscle contractions via cleavage of CP into creatine plus free phosphate. Free phosphate is thus able to regenerate ATP, recharging the ADP molecule into ATP, in a reaction catalyzed by muscular cytosolic creatine kinase (CK) [93]. The most common effect of creatine is to increase the muscle tissue stores of CP because a major portion of dietary/supplemented creatine is transported into the skeletal muscle tissue. During high-intensity muscle contractions, increased muscle CP stores can provide clear advantages to the contracting muscle [94]. As reviewed by Bemben and Lamont [8], first, increased CP generates more rapid energy thus reducing the dependence of the glycolytic metabolism. Second, increased free creatine is readily available for mitochondrial CK, which catalyzes the opposite reaction, resynthesizing CP from ATP in the mitochondria during recovering periods. Third, increased CP stores carry the potential to buffer H^+ ions formed during ATP hydrolysis and anaerobic glycolysis in the contracting muscle. Summed up, all supra-cited mechanisms of action could lead to an ergogenic effect that results in generating metabolites and increases the vasodilation/cellular swelling effect. Regarding the vasodilatory properties of creatine, it is not completely known if the mechanisms of action are due to its direct effects on vasodilation or a consequence of increased muscle metabolites/increased muscular work. Creatine monohydrate supplementation has been shown to diminish arterial stiffness and systolic blood pressure after resistance exercise performed with isokinetic

equipment [95]. Interestingly, immediately after maximal efforts, creatine supplementation has been shown to attenuate the increase in heart rate and this response seems to be partially due to a reduced muscle ammonia and lactic acid production [96]. When taken collectively, this information suggests that increased muscle metabolites may not be the main mechanism leading to increased vasodilation induced by creatine.

Creatine may pose a more direct effect on vasodilation and vascular function. de Moraes et al. [97] observed in healthy young adults that creatine supplementation improved systemic endothelial-dependent microvascular reactivity and increased skin capillary density and recruitment, both markers of increased microvascular function. These effects occurred independent of changes in the plasma levels of homocysteine, although creatine is also postulated to reduce homocysteine (Hcy) levels (creatine supplementation has been shown to change the methylation flux in the cell, reducing the conversion of methionine to homocysteine) [98]. However, little is known about the possible interactions between methyl balance modulation and lipid peroxidation when Hcy production is decreased by creatine supplementation. Homocysteine likely impairs vasodilation by promoting the expression of monocyte chemoattractant protein-1, increased reactive oxygen species production, activating NADPH oxidase, and reduced levels of superoxide dismutase [99,100]. Creatine was also tested in spontaneously hypertensive rats, which demonstrate increased Hcy levels. However, chronic creatine supplementation did not change blood pressure or cardiac structure parameters [101]. To the best of our knowledge, creatine supplementation carries the potential to increase vasodilation, but whether this specific effect translates to increased myocyte swelling and muscle hypertrophy is still unclear.

Supplements with osmotic properties: Creatine and betaine

Increased muscle creatine content can modify the osmotic pressure resulting in an increased intracellular influx of water to the cell, thereby enhancing the stored fluid volume of the myocyte. In other words, intramuscular creatine stores can promote water retention and, consequently, body weight gain. This effect was mainly observed after supplementation of 20 to 25 g/d for 5 to 7 d [102–104].

There are individuals who supplement creatine and associate the acute sensation of cellular swelling as indicator of effectiveness. Although it has been described to occur acutely [105], the current evidence supporting cellular swelling induced by creatine ingestion as a direct mechanism for muscle hypertrophy are lacking. However, there is a rationale that supports such a physiological phenomenon. As discussed previously, increased muscle water content is a trigger for protein synthesis, although caution should be taken when interpreting such information. Louis et al. [106] demonstrated that supplementation with 21 g/d of creatine for 5 d did not change the rates of muscle protein synthesis and degradation in the postabsorptive and postprandial states. Later, the same group showed similar results with the same supplementation protocol in combination with resistance exercise (20 sets × 10 reps of leg extension at 75% 1 RM) [107]. It is important to note that protein synthesis is a multifactorial phenomenon that depends on several stimuli and conditions (training level). Cell swelling is one of the stimuli that may contribute to protein synthesis. In this regard, it has been demonstrated that creatine supplementation significantly upregulates the expression of genes and protein contents of kinases involved in osmosensing and signal transduction [108]. Moreover, after 9 d of creatine supplementation, decreased whole-body protein breakdown [109] was observed in young men, similar to that observed in the supra-cited study infusing a hypo-osmotic solution to induce cell swelling [37,109]. During in vitro conditions absent of exercise, however, creatine supplementation does not seem to exert its effects predominantly via cellular swelling mechanisms. Both myofibrillar and sarcoplasmic protein synthesis increased in C2C12 muscle cells after 96 h of creatine treatment, however, increases in osmolarity (either intracellular or extracellular induced by β-alanine and mannitol, respectively) did not mimic any effects of creatine in increasing muscle protein synthesis, myosin heavy chain II content, differentiation, or myogenic fusion [91]. On the other hand, both free creatine and phosphocreatine increased after treatment, whereas the catabolism of phosphocreatine to produce creatine seems to be responsible for some of the cellular swelling effects during exercise [24]. Such results suggest that, in vivo, creatine's anabolic effects may be mediated by both direct (via the Akt-PKB pathway) or indirect (cell swelling related) mechanisms. In support of the direct myogenic theory, it has been demonstrated that creatine strongly influences satellite cell activation and posttranslational phosphorylation of proteins [108,110] and such effect may be responsible for the increased muscle hypertrohy observed in an animal model of compensatory hypertrophy [111]. Moreover, creatine supplementation increases the expression of mRNA coding for insulin-like growth factor [91] and increases mRNA expression of myogenic regulatory factor 4 in association with resistance training in human muscles [112].

Regarding the osmotic effects of creatine, a notion that has been propagated by coaches is that the change in water distribution could be harmful in some situations. For example, exposure to hot environments or heat loss induced by exercise (sweating), would lead to water losses mainly represented by extracellular volume, which would be affected by creatine supplementation inducing increased cellular hydration. However, this is mere speculation because the measurement of changes in intra and extracellular water volume with creatine consumption has not been demonstrated consistently. Powers et al. [12] conducted a randomized, double-blind, placebo-controlled study in which 16 men and 16 women ingested 25 g/d of creatine daily for 7 d and 5 g/d for 21 d and evaluated the muscle creatine concentration by biopsy (vastus lateralis muscle) and intra and extracellular fluid volume by deuterium oxide and sodium bromide dilution analyses. The authors demonstrated that the group supplemented with creatine showed higher total body water volume than the placebo group, indicating a cellular swelling effect. However, the distribution of water (intra and extracellular compartments) was not altered by supplementation. That is, creatine supplementation can promote body fluid retention but that does not result in lower extracellular fluid volume and, consequently, does not affect body temperature regulation or increase the incidence of muscle cramps. We conclude that although increases in intracellular water content and increases in body weight have been described after 3 d of creatine supplementation [105], prolonged increases in body water does not seem to be compartmentalized in favor of intra versus extracellular spaces. Thus, it is still unknown to what degree, if, at all, cellular swelling mechanisms are responsible for creatine's anabolic effects.

Betaine

Trimethylglycine (betaine) is a neutral zwitterionic compound, a methyl derivative of glycine, and is a naturally occurring by-product of sugar beet refinement extracted from molasses [113]. Betaine serves dual roles in human physiology: as a methyl donor in transmethylation and as an osmolyte maintaining fluid balance. Catabolism of betaine occurs in hepatic and renal mitochondria and involves a series of transmethylation reactions resulting in the transmethylation of Hcy to methionine via betaine–Hcy *S*-methyltransferase and the subsequent generation of dimethylglycine [114].

Elevated plasma Hcy is an independent risk factor in the development of vascular disease [115] and has been associated with

several metabolic diseases such as obesity, diabetes, and metabolic syndrome [116]. Hcy is a source of oxidative damage in endothelial tissue and has been shown to induce inflammatory cytokine expression and secretion in human monocytes via oxidative stress [100]. In healthy individuals, Hcy has been shown to reduce coronary microvasculature dilator function [117]. Betaine has been used to treat genetic homocysteinuria [118] and supplementation with 6 g/d for 6 and 12 wk has been shown to reduce fasting plasma Hcy concentrations in healthy individuals by 20% and 50%, respectively [119,120]. Atkinson et al. [121] demonstrated that an acute, modest dose (500 mg) of betaine in healthy individuals decreases fasting plasma Hcy and attenuates the Hcy rise for 24 h after a methionine load. By reducing plasma Hcy, betaine and creatine supplementation may enhance vasodilation and the potential for cellular swelling during resistance training; however, further research is required to test this hypothesis.

Betaine that does not participate in the transmethylation of Hcy is readily taken up by tissues and used as an organic osmolyte in the regulation of cell volume [113]. Betaine may protect sensitive metabolic pathways against cellular hypertonicity. Examples of metabolic pathways sensitive to cellular hydration include protein turnover, amino acid and ammonia metabolism, lipid and carbohydrate metabolism, pH regulation, and gene expression [32]. Because ion influx is limited owing to the destabilization of protein structures, enzymatic function, and polarization [94], metabolic/osmotic stress may increase betaine uptake [122] by skeletal muscle because betaine does not compromise protein nor enzymatic structure and has been shown to stabilize cellular metabolic function under conditions of hypertonic stress [123]. Betaine also has been labeled a “counteracting” solute for its role in enhancing protein stability and countering the denaturing effect of urea [124] and reductions in pH [125] in vitro. In particular, betaine has been shown to protect myosin ATPase and myosin heavy-chain proteins against denaturation by urea [126]. Finally, the affinity of troponin for Ca2+, and therefore force production, is negatively affected by reductions in protein hydration [127].

The osmoregulated betaine transporter is found in liver and skeletal muscle and readily takes up betaine to maintain a higher tissue to plasma betaine concentrations, thereby increasing cellular hydration [128]. Because external osmotic stress as a result of high-volume resistance exercise results in the cellular accumulation of organic osmolytes [11], it is possible that increased betaine uptake by skeletal muscle may enhance this cellular swelling. Supplemental betaine may thereby enhance hypertrophy by increasing intracellular water and providing a more hospitable environment for excitation-contraction coupling and protein synthesis. Six weeks of betaine supplementation has been shown to increase muscle mass and arm cross-sectional area in strength-trained men [129]. However, to our knowledge, the effects of betaine supplementation on myofiber hyperhydration and skeletal muscle water volume have yet to be investigated and thus require further study to test these hypotheses.

Conclusions

Dietary supplements carry the potential to increase muscle performance and muscle hypertrophy via several distinct mechanisms of action, herein categorized as secondary physiological factors. This is an important topic to be covered because, in some cases, traditional mechanisms of action do not seem to fully account for the proposed hypertrophic effects of many of the supplements discussed in this review. In this regard, preliminary and ongoing research has been conducted in the sports supplements field to try to better elucidate the mechanisms of actions behind these supplements. In the present review, supplements showing potential for increased muscle metabolite accumulation and vasodilatory effects (direct or indirect) or showing increased potential for muscle water retention were discussed. Although there is not yet consistency about the supra-cited theoretical assumption, it seems that performance-enhancing substances such as creatine, β-alanine, and citrulline malate all carry some potential ergogenic effect for inducing muscle hypertrophy via increased work output. Whereas metabolite production may be enhanced as a result of increased anaerobic work output, and this may lead to increased anabolic cell signaling activation indirectly via hyperemia, a cumulative effect has not always been observed. This is possible because supplements such as β-alanine and creatine also have intramuscular H^+-buffering properties. Studies have reported both increases and no difference in plasma lactate between supplement and placebo, likely because increased intracellular H^+ buffering reduces lactate efflux via the lactate/proton co-transporter, resulting in less plasma lactate measured [130]. To fully evaluate the relationship between metabolite production, muscle hyperemia, and muscle hypertrophy, will therefore require methodology that assesses intramuscular lactate production in real time, such as via Raman spectrography [131].

Increased vasodilation caused by supplements with vasodilator properties and its use in combination with antioxidants has been speculated to increase muscle blood flow and enhance the hyperemic/cell swelling response. Although evidence exists for increased blood flow and muscle performance, a more direct link between increased muscle blood flow and increased cell swelling or muscle hyperemia is still missing. Increases in the arterial blood flow may result in increased oxygen/nutrient delivery and metabolic waste products removal. However, if not accompanied by an increased fluid shift in the contracting muscles, it may not result in increased cell swelling and downstream anabolic signaling. Last, because creatine and betaine supplementation has been shown in humans and animals, respectively, to increase whole-body water content and intracellular water content, and creatine supplementation has been shown to increase osmosensing gene expression, increased cell swelling has been hypothesized as a potential mechanism that explains their anabolic properties. At the moment, however, muscle hypertrophy associated with cell swelling effects induced by both supplements remains to be investigated.

Although secondary physiological determinants are involved in the hypertrophic response of metabolically intense resistance training, most supplements presenting hypertrophic potential via these responses have not been directly tested. At this point, only partial mechanisms explaining secondary physiological determinants of muscle hypertrophy exists, with most studies only performed acutely or in animals or in vitro. Future research involving sports nutrition supplements should specifically address the link between vasodilation, enhanced anaerobic energy production, metabolite accumulation, and muscle hyperemia/cell swelling and muscle hypertrophy in association with resistance exercises. From these new findings, novel perspectives may be brought into focus and their relevance may be determined.

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