Current In Vitro Testing of Bioactive Peptides Is Not Valuable\textsuperscript{1,2}

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Abstract
Dietary peptides have been suggested to possess biological activity in vivo and could affect cardiovascular disease parameters, based on data derived from in vitro experiments. Isolated peptides are often tested in in vitro cellular assays or on heterologously expressed molecular target proteins. The stimulatory or inhibitory effect on target proteins in vitro has often been used as the justification to test these compounds directly in vivo. Unfortunately, this research approach has an inherent flaw. It neglects the poor absorption, distribution, metabolism, and excretion (ADME) properties of peptides resulting in low peptide bioavailability. Because peptides are prone to extensive hydrolysis in the gastrointestinal tract by stomach, small intestinal, and brush border peptidases, most of them do not reach the absorption stage in the duodenum and jejunum. Therefore, a valid research approach should include the demonstration of stability of the peptide toward luminal and brush border peptidases and evaluate its ADME properties. Surprisingly, only very few animal and human studies determined absolute concentrations and kinetics of bioactive peptides. These studies have shown the presence of selected peptides in plasma samples at pico- and nanomolar concentrations with fast elimination kinetics in the minute range. For the correct interpretation of results, it is advised that researchers refer to the data currently available concerning bioavailability and ADME properties in humans. Two mandatory criteria for future in vitro studies investigating potential biological activities of peptides should be using physiologically relevant concentrations and times. J. Nutr. 140: 117–118, 2010.

Small endogenous peptides, such as peptide hormones and signaling peptides, present in the systemic circulation have strong effects on human physiology. It is hence a very appealing idea to develop tailor-made small peptides targeted at specific receptors and enzymes. This concept has been successfully applied in pharmacological approaches where, e.g., synthetic insulin and gonadotropin-releasing hormone 1 are first lines of treatment for diabetes mellitus and hypogonadism, respectively. The same concept has gained increasing interest from academia and food industries where it is reasoned that certain dietary peptides could also be potentially used as bioactive ingredients in functional foods. Dietary proteins have been shown to contain sequences of peptides, partially similar to those found in endogenous peptides, with hormonal or neuronal functions. These exogenous dietary peptides are proposed to exert physiological effects by acting either agonistically or antagonistically on the same targets as their endogenous counterparts. Based on these observations, many peptides derived from common food proteins have been proposed to influence several different physiological parameters, e.g., blood pressure, insulin and glucose homeostasis, plasma cholesterol concentrations, and immune function, among which those with potential antihypertensive activity have been studied the most (1). Data from observational studies supported this hypothesis by showing an inverse association between dairy consumption and blood pressure independent of calcium (2,3), suggesting that milk protein may play a critical role. Ideally, one would aim to confirm the findings from observational data in human intervention studies. However, because these studies are expensive and tedious to conduct, initial in vitro experiments are often performed to identify potential bioactive peptides before committing to full-blown human trials. For example, a huge variety of peptides derived from various dietary protein sources have been examined in cellular assays for their inhibitory properties on angiotensin-I converting enzyme (ACE) activity (4), one of the key enzymes in regulating blood pressure, and several food-grade peptides such as the tripeptides Ile-Pro-Pro and Val-Pro-Pro have been found to decrease, in vitro, the conversion of angiotensin I to the vasoconstrictor angiotensin II. These peptides displayed moderate, reversible ACE inhibitory activity in the micromolar range (5).

The hypothesis to use ACE inhibitory peptides for modulating blood pressure is very appealing, because a similar mechanism of action has been shown to be effective in lowering blood pressure using potent pharmaceutical ACE inhibitors, although those exhibit strong inhibition constants for ACE in the low

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nanomolar range (6). Many food-derived peptides have also been proposed to possess immunomodulatory activity. In various in vitro test systems, such as phagocytosis activity assays, Ig production, and lymphocyte proliferation tests, a number of casein-derived peptides were shown to have immune-enhancing effects when applied at micro- and even millimolar concentrations. However, data from human studies demonstrating that dietary peptides inhibit ACE or have direct immune-enhancing properties are still lacking.

Human tissues are exposed to bioactive peptides via the systemic circulation. However, before they reach it, peptides are substantially hydrolyzed during small intestinal passage and absorption. The human digestive system is capable of handling a wide range of protein sources and the cascade of gastrointestinal proteolytic and peptidolytic enzymes very efficiently degrades proteins from their quaternary structure into single amino acids. Although substantial amino acid absorption occurs in the form of di- and triptides at the apical side of enterocytes, efflux of intact peptides via the basolateral membrane into the general circulation seems to be negligible (7). In addition, vascular endothelial tissue peptides and soluble plasma peptides further contribute to peptide hydrolysis. As a consequence, for most peptides, the plasma half-life is limited to minutes as shown for endogenous peptides such as angiotensin II and glucagon-like peptide 1 (8). The previously mentioned severely limited absorption, combined with their rapid elimination, will therefore result in extremely low bioavailability for most natural peptides under physiological conditions. Despite the fact that selected peptides, such as C-terminal proline-containing peptides, exhibit distinct stability toward luminal peptidases, they are subject to brush border and cytosolic peptidases and only minor fractions will reach the systemic circulation. Therefore, knowing the plasma concentrations and kinetics of orally administered peptides is essential for planning meaningful in vitro studies assessing the bioactivity of dietary peptides. We consider it to be a flaw in most of the research strategies that peptides are first screened in vitro against potential targets and then in a next step directly tested in vivo to confirm efficacy. In our view, it is only valid to propose in vivo efficacy for bioactive peptides when the peptide exhibits reasonable proteolytic stability and physiologically relevant absorption, distribution, metabolism, and excretion (ADME) profiles.

We have studied this in more detail in animals and humans with the peptide prototypes Ile-Pro-Pro and Val-Pro-Pro. These peptides are known to possess high proteolytic stability, but the absolute bioavailability of the peptides determined in pigs was below 0.1%, with an extremely short elimination half-life ranging from 5 to 20 min (9) and in humans, maximal plasma concentration did not exceed high picomolar concentrations. This calculation did not take into account plasma protein binding effects, which could further reduce the availability of the peptide to reach its target protein (10). At the moment, there is no scientific evidence that other small peptides, originating from dietary sources, have substantially improved absorption or plasma clearance profiles, which could result in an acceptable bioavailability or even very transiently high, free plasma concentrations. ADME properties may be different only under certain pathophysiological conditions such as end stage renal disease.

Overall, the strictly limited absorption, as demonstrated in these studies, makes it almost impossible to link dietary peptides as agonists or antagonists on cellular targets in vivo. In addition, the short elimination half-life in the minute range would result in only a short duration of the effect, if any, and would require frequent repeated dosing. Certainly, for the functional food targets proposed, such as inhibition of ACE and immune stimulation, reasonable slow plasma kinetics of the bioactive peptide would be required to result in a sustained effect.

The majority of in vitro studies, for identification of bioactive peptides, were conducted at high micromolar and even millimolar concentrations and in some examples with incubation times lasting as long as 24 h. These conditions, given the aforementioned peptide ADME properties, are neither realistic nor physiologically relevant. In vitro screening should be performed using a concentration range with 1 or 2 orders of magnitude above or below the actual plasma concentrations. At best, effects should be observable within minutes up to within a few hours to correlate to end effects in vivo. Applying such an experimental “filter,” based on true low oral bioavailability and fast pharmacokinetics, in the search for and evaluation of existing functional peptides will result in more relevant hits and prevent disappointments when making the step from preclinical to clinical research. We hope that mentioning this issue will alert unsuspecting researchers that there are pitfalls and serious limitations in the direct transfer of data obtained from in vitro experiments with peptides to in-human responses. The aforementioned reasons most likely contribute to the several “no effect” publications on dietary peptide intervention studies that were aimed at postabsorptive targets.

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Literature Cited

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